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Rho-kinase regulates adhesive and mechanical mechanisms of pulmonary recruitment of neutrophils in abdominal sepsis

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ABSTRACT

We hypothesized that Rho-kinase signaling plays a role in mechanical and adhesive mechanisms of neutrophil accumulation in lung. Male C57BL/6 mice were treated with the Rho-kinase inhibitor Y-27632 prior to cecal ligation and puncture (CLP). Lung levels of myeloperoxidase (MPO) and histological tissue damage were determined 6 h and 24 h after CLP. Expression of Mac-1 and F-actin formation in neutrophils were quantified by using flow cytometry 6 h after CLP. Mac-1 expression and F-actin formation were also determined in isolated neutrophils up to 3 h after stimulation with CXCL2. Labeled and activated neutrophils co-incubated with Y-27632, an anti-Mac-1 antibody and cytochalasin B were adoptively transferred to CLP mice. Y-27632 reduced the CLP-induced pulmonary injury and MPO activity as well as Mac-1 on neutrophils. Neutrophil F-actin formation peaked at 6 h and returned to baseline levels 24 h after CLP induction. Rho-kinase inhibition decreased CLP-provoked F-actin formation in neutrophils. CXCL2 rapidly increased Mac-1 expression and F-actin formation in neutrophils. Co-incubation with Y-27632 abolished CXCL2-induced Mac-1 up-regulation and formation of F-actin in neutrophils. Notably, co-incubation with cytochalasin B inhibited formation of F-actin but did not reduce Mac-1 expression on activated neutrophils. Adoptive transfer experiments revealed that co-incubation of neutrophils with the anti-Mac-1 antibody or cytochalasin B significantly decreased pulmonary accumulation of neutrophils in septic mice. Our data show that targeting Rho-kinase effectively reduces neutrophil recruitment and tissue damage in abdominal sepsis. Moreover, these findings demonstrate that Rho-kinase-dependent neutrophil accumulation in septic lung injury is regulated by both adhesive and mechanical mechanisms.

1. Introduction

Intestinal perforation is a major cause of mortality in intensive care units and poses a significant challenge for clinicians (Cohen, 2002; Heyland et al., 2000; Martin et al., 2003). Dissemination of bacteria and their toxins in the abdominal cavity provokes local formation of various pro-inflammatory compounds, which subsequently leak into the circulation, where these mediators activate circulating neutrophils causing a systemic inflammatory response (Aird, 2003). The lung is the most sensitive and clinically important end organ in abdominal sepsis. It is widely held that pulmonary recruitment of neutrophils is a rate-limiting step in septic lung injury. For example, neutrophil depletion or targeting specific adhesion molecules, such as PSGL-1 and Mac-1 are effective ways to protect against sepsis-induced lung damage (Asaduzzaman et al., 2009; Zhang et al., 2011). The recruitment process of neutrophils in the lung is more complex and far less studied than in other organs. Under homeostatic conditions, most neutrophils, which have a diameter larger than that of pulmonary capillaries, must deform in order to pass through the pulmonary microcirculation (Motosugi et al., 1996). Thus, any reduction in their deformability would promote mechanical sequestration of neutrophils in the lung capillaries (Worthen et al., 1989). Considered together, neutrophil accumulation may depend on both adhesive and mechanical factors in the lung. On one hand, adhesion molecules, such as selectins and integrins may support leukocyte–endothelium interactions in the pulmonary microvasculature. On the other hand, activated leukocytes may trigger cytoskeletal changes, including polymerization of F-actin, resulting in cell stiffening and mechanical trapping in the narrow capillaries in the lung. Whether adhesive and mechanical mechanisms of neutrophil accumulation operate in parallel and/or sequentially are not known.

Extracellular stress, including ischemia and infection, activates cellular signaling cascades converging on specific transcription factors regulating gene expression of inflammatory mediators. This intracellular signal transmission is largely regulated by kinases phosphorylating down-stream targets (Itoh et al., 1999). For example, small (~21 kDa)

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guanosine triphosphatases of the Ras-homologus (Rho) family and one of their effectors, Rho-kinase, are known to act as molecular switches controlling several critical functions, including, cell adhesion and contraction, migration, reactive oxygen species formation and oncogenic transformation (Alblas et al., 2001; Itoh et al., 1999; Slotta et al., 2006). Interestingly, Rho-kinase inhibitors have been reported to attenuate ischemia/reperfusion and endotoxemic injury in the liver (Slotta et al., 2008) as well as protecting against tissue fibrosis (Kitamura et al., 2007), cholestasis (Laschke et al., 2010), cerebral and intestinal ischemia (Santen et al., 2010; Shin et al., 2007). However, the role of the Rho-kinase signaling in regulating pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis is not known. Moreover, the influence of Rho-kinase inhibition on adhesive and mechanical aspect of sepsis-induced neutrophil accumulation in the lung remains elusive.

Based on these considerations, the aim of the present study was to define the functional role of Rho-kinase signaling in regulating F-actin polymerization and Mac-1 expression in neutrophils as well as their role in the regulation of pulmonary recruitment of neutrophils and tissue damage in sepsis. For this purpose, we used a model of polymicrobial sepsis based on intestinal perforation in mice.

2. Materials and methods

2.1. Animals

Specific-pathogen-free male C57BL/6 mice (20 to 25 g) were housed in filter-top cages under standardized laboratory conditions. Mice were with a 12 h light/12 h dark diurnal cycle with food and water *ad libitum*. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Animals were anesthetized by administration of 7.5 mg (i.p.) ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg (i.p.) xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

2.2. Experimental protocols

Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) as described previously (Asaduzzaman et al., 2008). Shortly, through laparotomy incision, the exposed cecum was filled with feces by milking stool backward from the ascending colon, and a ligature was placed below the ileocecal valve. The cecum was soaked with phosphate-buffered saline (PBS; pH 7.4) and punctured twice with a 21-gauge needle. The cecum was then pushed back into the abdominal cavity and the incision was sutured. To determine the role of Rho-kinase, vehicle (PBS) or the Rho-kinase inhibitor, Y-27632 {(R)-(+)-N-(4-pyridyl)-4-(1-aminoethyl} cyclohexanecarboxamide; (Calbiochem, San Diego, USA), was given (5.0 mg/kg) i.p. 30 min before CLP induction. The dose of 5 mg/kg was chosen based on a previous study (Awla, et al., 2011). Sham mice underwent the same surgical procedures, but the cecum was neither ligated nor punctured. The mice were then returned to their cages and provided food and water. Animals were re-anesthetized 3, 6, 12 and 24 h after CLP induction. The lung was perfused with PBS, left lung was fixed in formaldehyde for histology, and the remaining lung tissue was weighed, snap-frozen in liquid nitrogen, and stored at −80°C for later myeloperoxidase (MPO) assays as described below.

2.3. Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turk's solution (0.2 mg gentian violet in 1 ml glacial acetic acid; 6.25% vol/vol) in a 1:20 dilution. Leukocytes were identified and counted as monomorphonuclear (MNL) and polymorphonuclear (PMNL) leukocyte cells in a Burker chamber.

2.4. MPO activity

The enzyme MPO is abundant in neutrophils and has been used as reliable marker for detection of neutrophil accumulation in inflamed tissue and was quantified as described previously (Bradley et al., 1982). In brief, frozen lung tissue was thawed and homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter of 540 nm; 25 °C). Values were expressed as MPO units per gram of tissue.

2.5. Histology

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six-micrometer sections were stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a pre-existing scoring system as described (Borzone et al., 2007), including size of alveolar spaces , thickness of alveolar septas, alveolar fibrin deposition and neutrophil infiltration, graded on a 1 (absent) to 4 (extensive) scale. The total injury score was expressed as the sum of the four scores.

2.6. Flow cytometry

For detection and analysis of Mac-1 expression on circulating neutrophils, blood was collected into syringes containing 1:10 acid citrate dextrose (22.0 g/L sodium citrate; 7.3 g/L citric acid, anhydrous; and 24.5 g/L dextrose) 3, 6, 12 and 24 h after CLP induction. Blood samples were incubated with an anti-CD16/CD32 antibody (10 min at room temperature) blocking Fcγ III/II receptors to reduce non-specific labeling and then incubated with PE-conjugated anti-Gr-1 (Clone RB6-8C5, rat IgG2b, eBioscience, San Diego, USA), and FITC-conjugated anti-Mac-1 (Clone M1/70, integrin α_M chain, rat IgG2b) antibodies. To determine the distribution of F-actin content within neutrophils, another set of samples was permeabilized by L-lysophosphatidylcholine (LPC, lysolecithin, 1-O-acyl-sn-glycero-3-phophocholine, L-lysophosphatidylcholine-gamma-Oacyl; Sigma, St. Louis , MO, USA) and then stained with BODIPY FL phallicidin (Molecular Probes, Invitrogen, Eugene, USA) and APC-conjugated anti-Gr-1 (Clone RB6-8 C5, rat anti-mouse Ly-6G and Ly-6C) antibodies (all antibodies except those indicated were purchased from BD Biosciences Pharmingen, San Jose, CA, USA). Cells were fixed and erythrocytes were lysed using FACS lysing solution (BD Biosciences Pharmingen, San Jose, CA, USA) and then neutrophils were recovered following centrifugation. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and a viable gate was used to exclude dead and fragmented cells.

2.7. In vitro studies

Neutrophils were freshly isolated from healthy mice by aseptically flushing the bone marrow of femurs and tibias from healthy mice by using Ficoll-Paque™ Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was more than 70% as assessed in a haematocytometer. Leukocytes were then re-suspended in PBS to 10×10^6/ml and co-incubated with 300 ng/ml recombinant mouse CXCL2/MIP2 (R&D Systems, Inc., Minneapolis, USA) for different time points. Two set of samples were pre-incubated with Y-27632 (100 μM/100 μl) 20 min before activation by CXCL2 for measuring F-actin content within neutrophils and Mac-1 expression.

Cells are permeabilized, stained and fixed as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur). Mac-1 expression on neutrophils and F-actin content within neutrophils were measured in separate experiments. Lastly effects of cytochalasin B 10 μM (cytochalasin B isolated from Drechslera dematioidea; Sigma, Sweden) on F-actin and Mac-1 were checked in two different sets of experiment.

2.8. Adoptive transfer of neutrophils

Again bone marrow leukocytes were freshly extracted from healthy mice by using Ficoll-Paque™ as described above. Leukocytes were then re-suspended in PBS to 10×10^6/ml and co-incubated with 300 ng/ml recombinant mouse CXCL2 for 10 min and 180 min separately; furthermore leukocytes were pre-incubated with cytochalasin B 10 μM 30 min and anti-Mac-1 (Purified anti-mouse CD11b, NA/LE, from BD Biosciences) for 15–20 min at room temperature before challenge with CXCL2 co-incubation. Samples were stained with 20 μM CFDA-SE (carboxyfluorescein diacetate-succinimidyl ester, Invitrogen, Paisley, UK) and for 1 h at 37 °C. CFDA-SE passively diffuses into cells and is non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent ester. Two million labeled neutrophils were injected intravenously into mice immediately prior to CLP, then 4 h after CLP induced, lungs were harvested, minced, and digested for 1 h at 37 °C in buffer containing 20 U/ml collagenase A (Sigma). Single-cell suspensions were obtained by straining the digested tissue through a 40-μm mesh. Cells were labeled with an APC-labeled anti-Gr-1 antibody and fixed as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur). Lung recruitment of transferred neutrophils were quantified by dividing the number of $CFDA^+/Gr\text{-}1^+$ cells by the number of $CFDA^-/Gr\text{-}1^+$ cells in the lung extracts.

2.9. Statistics

Data are presented as mean values ± S.E.M. (Standard error of the mean). Statistical evaluations were performed by using Kruskal–Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunnett's method). $P<0.05$ was considered significant and n represents the number of animals in each group.

3. Results

3.1. Rho-kinase activity regulates lung tissue damage

CLP caused significant pulmonary damage, characterized by severe destruction of pulmonary tissue microstructure, extensive edema of interstitial tissue, necrosis and massive infiltration of neutrophils (not shown). Quantification of the morphological damage showed that CLP enhanced the lung injury score and that pretreatment with the Rho-kinase inhibitor Y-27632 significantly reduced the lung injury score in CLP mice (Fig. 1A; $P<0.008$ vs. vehicle + CLP, $n=5$). Moreover, leukocytopenia was observed 24 h after CLP induction (Table 1). For example, the number of neutrophils decreased by 53% 24 h after CLP (Table 1; $P<0.05$ vs. sham, $n=5$). Notably, this CLP-induced neutropenia was significantly decreased in mice pretreated with Y-27632 (Table 1; $P<0.05$ vs. vehicle + CLP, $n=5$).

3.2. Mac-1 expression and neutrophil accumulation are regulated by Rho-kinase activity

Accumulation of neutrophils in the lung was quantified by analyzing levels of myeloperoxidase (MPO), an indicator of neutrophils in the lung. It was found that CLP increased MPO activity in the lung by 11-fold (Fig. 1B; $P<0.008$ vs. sham, $n=5$). Notably, Rho-kinase inhibition reduced MPO activity in the lung by 54% in septic mice (Fig. 1B; $P<0.008$ vs. vehicle + CLP, $n=5$). Mac-1 expression was increased on

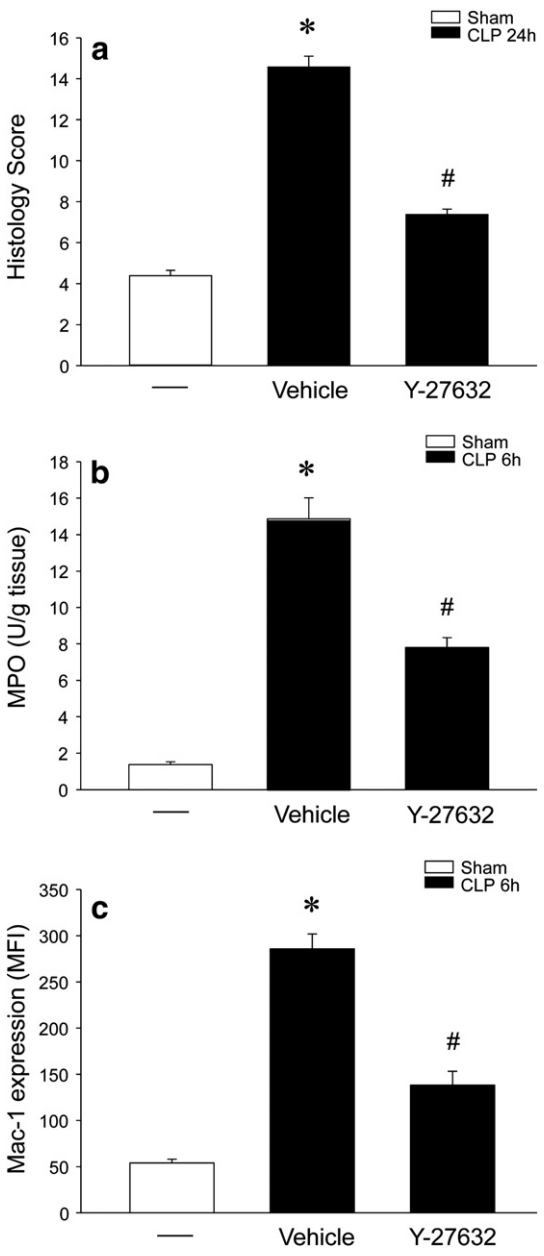

Fig. 1. (A) Evaluation of lung injury at 24 h as described in Materials and methods. (B) Lung MPO activity, a marker of neutrophils in the lung 6 h post-CLP. (C) Rho-kinase activity regulates Mac-1 expression on neutrophils. Sham animals served as a negative control and separate groups of mice were pretreated with vehicle and Y-27632 before operation. Data represents mean ± S.E.M. and $n=5$. $^*P<0.05$ vs. sham and $^\#P<0.05$ vs. vehicle + CLP.

Table 1
Systemic leukocyte differential counts.

	MNL	PMNL	Total
Sham	4.8 ± 0.1	1.5 ± 0.05	6.3 ± 0.1
Vehicle + CLP	1.2 ± 0.1[a]	0.4 ± 0.1[a]	1.6 ± 0.1[a]
Y-27632 + CLP	1.4 ± 0.1[a]	0.9 ± 0.1[a,b]	2.3 ± 0.2[a,b]

Blood was collected from vehicle and Y-27632 treated mice exposed to cecal ligation and puncture (CLP) for 24 h as well as sham-operated animals. Cells were identified as monomorphonuclear leukocytes (MNLs) and polymorphonuclear leukocytes (PMNLs). Data represents mean ± S.E.M. x 10^6 cells/ml and $n=5$.
[a] $P<0.05$ vs. sham.
[b] $P<0.05$ vs. vehicle + CLP.

the surface of neutrophils in CLP animals, indicating that circulating neutrophils were activated in this sepsis model. Inhibition of Rho-kinase activity markedly antagonized CLP-induced up-regulation of Mac-1 on the surface of neutrophils. MFI (mean fluorescence intensity) values of Mac-1 on neutrophils decreased from 285 ± 16 down to 138 ± 15 in CLP mice pretreated with Y-27632, corresponding to a 63% reduction (Fig. 1C; $P<0.029$ vs. vehicle + CLP, $n=5$).

3.3. Rho-kinase activity regulates neutrophil formation of F-actin

We first analyzed the F-actin content of neutrophils at different time points after CLP induction. F-actin formation in neutrophils increased rapidly in septic animals, *i.e.* MFI values of F-actin increased from 122 ± 3 at baseline to 264 ± 24 3 h after CLP (Fig. 2A; $P<0.008$ vs. sham, $n=5$). Maximum levels of F-actin were observed 6 h after induction of CLP when MFI values of F-actin reached 270 ± 26 (Fig. 2A; $P<0.008$ vs. sham, $n=5$). F-actin formation in neutrophils returned to baseline levels 24 h after CLP induction (Fig. 2A). Next we asked whether inhibition of Rho-kinase activity might influence F-actin formation in neutrophils. Administration of Y-27632 decreased the CLP-provoked formation of F-actin in neutrophils (Fig. 2B). Indeed, inhibition of Rho-kinase activity reduced neutrophil formation of F-actin by 68% in septic mice (Fig. 2C; $P<0.001$ vs. vehicle + CLP, $n=5$).

3.4. CXCL2-induced F-actin formation and Mac-1 expression in vitro

F-actin formation and Mac-1 expression were determined in isolated neutrophils after stimulation with CXCL2, which is a potent activator of neutrophils. Challenge with CXCL2 caused a rapid increase in F-actin formation in neutrophils peaking at 10 min and returning to baseline levels after 180 min (Fig. 3A). Neutrophil expression of Mac-1 also peaked at 10 min but remained elevated compared to baseline values 180 min after stimulation with CXCL2 (Fig. 3B). It was found that co-incubation of neutrophils with Y-27632 abolished CXCL2-induced formation of F-actin and expression of Mac-1 in neutrophils (Table 2). Interestingly, co-incubation of neutrophils with cytochalasin B, a well-known inhibitor of F-actin polymerization, also abolished CXCL2 triggered F-actin formation (Table 2; $P<0.008$ vs. vehicle + CXCL2, $n=5$) but had no effect on surface expression of Mac-1 (Table 2) on neutrophils.

3.5. Adhesive and mechanical mechanisms of neutrophil accumulation

In order to discriminate the influence of adhesive and mechanical mechanisms in the accumulation process of neutrophils in septic lung damage, we performed adoptive transfer of CFDA-labeled and CXCL2 activated neutrophils co-incubated with or without the anti-Mac-1 antibody and cytochalasin B. In line with the kinetic experiments showing maximal expression of Mac-1 and F-actin formation in neutrophils 10 min after challenge with CXCL2, we observed that homing of adoptively transferred neutrophils was maximal when the cells had been stimulated with CXCL2 for 10 min (Fig. 4A; $P<0.010$ vs. sham, $n=6$). It was found that homing of CFDA-labeled and activated neutrophils to the lung in CLP mice were markedly reduced when neutrophils were co-incubated with the anti-Mac-1 antibody (Fig. 4B). Notably, co-incubation of labeled and activated neutrophils with cytochalasin B, which had no effect on Mac-1 expression, also significantly decreased pulmonary accumulation of these cells in CLP animals (Fig. 4C; $P<0.001$ vs. vehicle + CXCL2, $n=5$).

4. Discussion

It is widely held that systemic activation and pulmonary accumulation of neutrophils are key features in sepsis. However, the signaling pathways regulating neutrophil activation remain elusive. In the present study, we show that the Rho-kinase inhibitor Y-27632 greatly

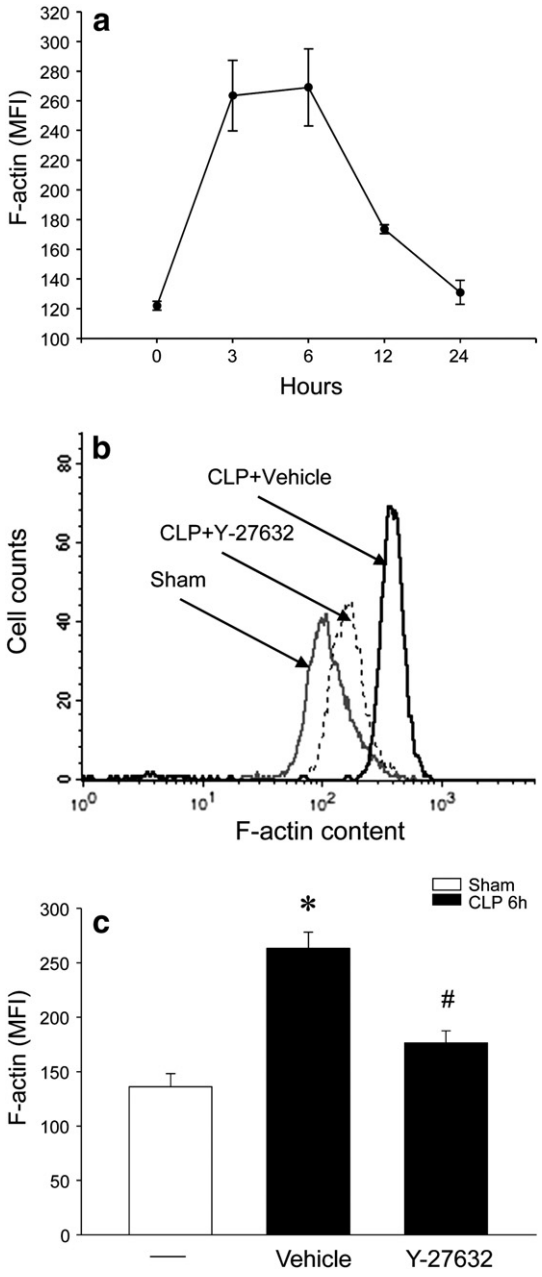

Fig. 2. CLP rapidly increased F-actin content of neutrophils and reached maximum levels 6 h after CLP. F-actin content (MFI) of the neutrophils shown on the Y-axis against time after CLP induction on the X-axis (a). Pre-treatment with Y-27632 significantly reduced F-actin content at 6 h CLP (b) and (c). Data represents mean ± S.E.M. and $n=5$. $^{*}P<0.05$ vs. sham and $^{\#}P<0.05$ vs. vehicle + CLP.

reduced pulmonary levels of MPO, an indicator of neutrophils, suggesting that Rho-kinase controls neutrophil accumulation in septic lung injury. Moreover, inhibition of Rho-kinase activity not only decreased pulmonary neutrophilia but also attenuated lung tissue damage in abdominal sepsis. Considering the close relationship between neutrophil recruitment and tissue damage in septic pulmonary injury (Asaduzzaman et al., 2008) it may be proposed that part of the lung protective effect of inhibiting Rho-kinase signaling is related to the reduced accumulation of neutrophils in the lung. These findings are in line with two previous studies showing that Rho-kinase inhibition can reduce tissue injury in the liver and lung triggered by endotoxin (Lomas-Neira et al., 2006; Thorlacius et al., 2006). Although toxin-based models are frequently used, they are more artificial and do not necessarily resemble the complex events and course in polymicrobial sepsis in terms of

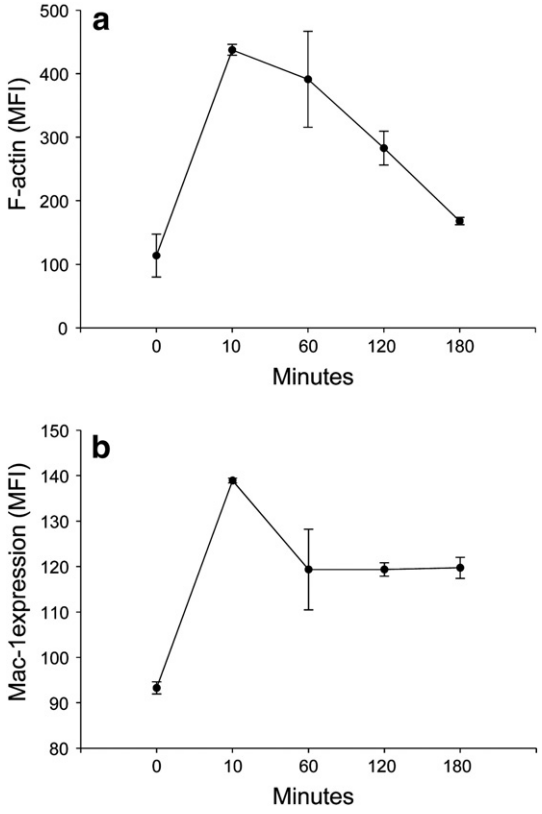

Fig. 3. *In vitro* kinetics of F-actin content (MFI) and Mac-1 expression (MFI). Shown on the Y-axis against time after CXCL2 activation on the X-axis. F-actin peaked at 10 min and reduced at 180 min (a). Mac-1 expression peaked at 10 min again but remained relatively high at 180 min (b). Data represents mean ± S.E.M. and $n = 5$.

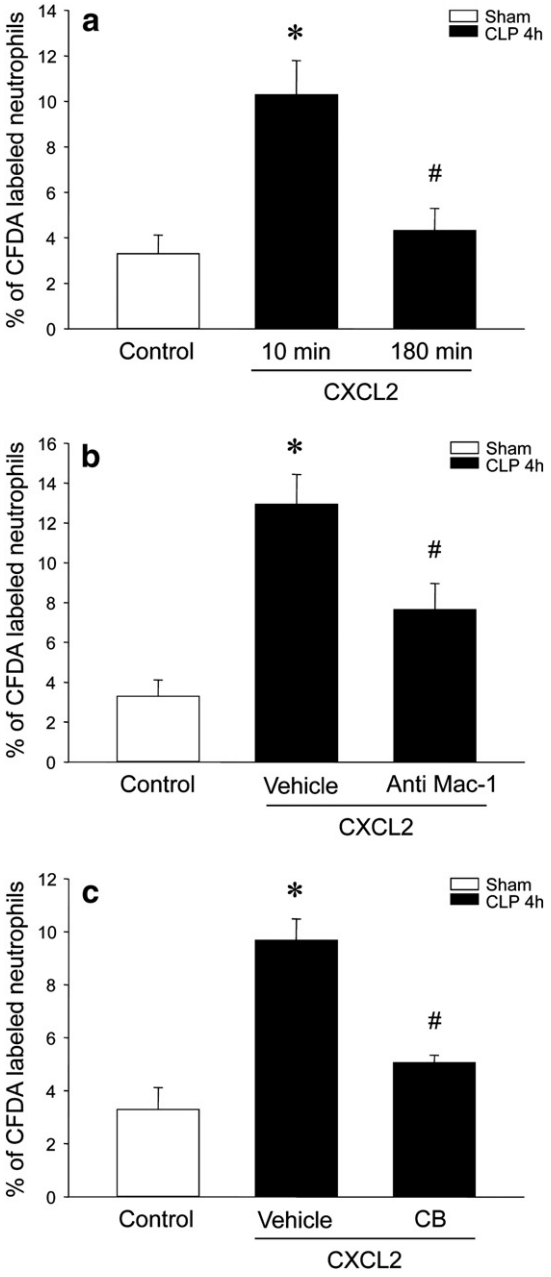

Fig. 4. (a) Adoptive transfer of CFDA-labeled neutrophils. Bone marrow neutrophils were labeled with CFDA and 2×10^6 neutrophils and incubated with CXCL2 for 10 min and 180 min and then adoptively transferred to CLP mice, Data represents mean ± S.E.M. and $n = 6$. $^*P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. 10 min + CLP. CFDA-labeled neutrophils activated with CXCL2 for 10 min were co-incubated with an anti-Mac-1 (b) or cytochalasin B (c) prior to injection. Accumulation of transferred neutrophils was determined by quantifying the number of neutrophils (Gr-1 + cells) in lung labeled with CFDA divided by the total number of neutrophils (Gr-1 + cells). Data represents mean ± S.E.M. and $n = 5$. $^*P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. vehicle + CXCL2.

cytokine responses as well as vascular and metabolic changes (Klintman et al., 2004b; Remick et al., 2000; Wichterman et al., 1980). Moreover, different toxins activate the host immune system in a distinctly different manner. For example, LPS has been shown to be a potent activator of macrophages and stimulates TNF-α production (Ulevitch et al., 1990; Wright et al., 1990) whereas superantigens do not provoke clear-cut TNF-α formation and activates primarily T-lymphocytes causing FasL-dependent apoptosis (Klintman et al., 2004a). Nonetheless, considered together with our present findings, it may be forwarded that Rho-kinase signalling is a key feature in acute lung injury.

Leukocyte recruitment in the lung is far less studied and appears to be much more complex than that in other organs. One reason for this is that the spherical diameter of the capillaries in the lung is smaller (6 μm) than that of neutrophils (7 μm) which forces neutrophils to deform and make them prone to mechanical trapping when passing through the narrow lung capillaries (Motosugi et al., 1996). Thus, cytoskeletal rearrangements causing any reduction in neutrophil deformability will increase their sequestration in the pulmonary microvasculature. This concept of F-actin-mediated stiffening and mechanical trapping of neutrophils in the lung has been confirmed in a number of studies (Downey et al., 1991; Frank, 1990; Saito et al., 2002). Therefore, it was of great interest to study F-actin formation in circulating neutrophils in septic mice herein. We observed that F-actin markedly increased in circulating neutrophils in mice with ongoing abdominal sepsis. Moreover, this sespis-induced neutrophil F-actin formation was abolished by administration of Y-27632, indicating that polymerization of F-actin in neutrophils is regulated by Rho-kinase signaling in abdominal sepsis. In contrast, Tasaka et al. (2005) reported that neutrophil formation of F-actin triggered by fMLP, a peptide from *Escherichai coli* bacteria, was insensitive to treatment with Y-27632. The reason behind these apparent discrepancies is not known. However, the mechanisms of neutrophil stiffening in polymicrobial sepsis *in vivo* is likely different from that induced by bacterial proteins *in vitro*. For instance, it is well known that mechanisms of neutrophil stiffening differs signicantly between different stimulus, such as fMLP (Saito et al., 2002) and LPS (Erzurum et al., 1992). In addition, our findings are in line with convincing *in vitro* data showing that the Rho-kinase signaling pathway controls polymerization of F-actin in neutrophils (Chodniewicz and Zhelev, 2003). A common observation in systemic inflammation is a marked decrease in the number of circulating neutrophils due to mechanical sequestration in the lung (Andonegui et al.,

Table 2
Mac-1and F-actin expression in neutrophils.

	Mac-1 expression (MFI)	F-actin expression (MFI)
PBS	395 ± 1.8	176 ± 1.4
CXCL2	1301 ± 11.3[a]	415 ± 3.7[a]
CXCL2 + Y-27632	746 ± 10.4[b]	237 ± 16.2[b]
CXCL2 + CB	1530 ± 10.8	304 ± 14.5[b]

F-actin polymerization and Mac-1 expression in isolated bone marrow neutrophils were determined 10 min after challenge with CXCL2. Neutrophils were co-incubated with Y-27632 and cytochalasin B (CB). Data represent mean ± S.E.M. and $n = 5$.
[a] $P<0.05$ vs. PBS.
[b] $P<0.05$ vs. CXCL2.

2003). Indeed, we also found that systemic neutrophil counts were significantly reduced in CLP mice. It is therefore interesting to note that administration of Y-27632 reduced the CLP-induced decrease in circulating neutrophils, which further lends support to the concept that Rho-kinase signaling regulates neutrophil stiffening in abdominal sepsis.

Besides mechanical trapping, leukocytes are also recruited in the lung by specific adhesion molecules expressed on leukocytes and endothelial cells. For example, it has been shown that PSGL-1, LFA-1 and Mac-1 on neutrophils and ICAM-1 on endothelial cells support pulmonary accumulation of neutrophils in abdominal sepsis (Asaduzzaman et al., 2008, 2009; Hildebrand et al., 2005). Herein, we found that expression of Mac-1 was markedly increased on the surface of circulating neutrophils in septic mice. Moreover, administration of Y-27632 abolished sepsis-induced Mac-1 expression on neutrophils, indicating that Rho-kinase regulates Mac-1 up-regulation on neutrophils in abdominal sepsis. Considered together, our findings suggest that Rho-kinase may control sepsis-evoked neutrophil recruitment in the lung via both adhesive and mechanical mechanisms. In order to better define the relative role of adhesive and mechanical mechanisms in this Rho-kinase-dependent neutrophil recruitment in the lung, we stimulated isolated neutrophils with CXCL2 *in vitro*. We observed that CXCL2 increased Mac-1 expression and F-actin formation in neutrophils in a time-dependent manner peaking 10 min after challenge. It was found that Y-27632 reduced CXCL2-induced neutrophil up-regulation of Mac-1 as well as F-actin formation. In contrast, cytochalasin B, a well-known inhibitor of F-actin polymerization (Cooper, 1987), abolished F-actin formation but had no effect on Mac-1 expression on neutrophils in response to CXCL2 stimulation. Next, we adoptively transferred isolated and labeled neutrophils activated with CXCL2 for 10 min to mice with ongoing abdominal sepsis and determined their accumulation in the lung. We found that co-incubation of isolated neutrophils with an antibody directed against Mac-1 markedly reduced pulmonary recruitment of these neutrophils in septic animals. Interestingly, co-incubation of isolated neutrophils with cytochalasin B, which had no effect on Mac-1 expression but abolished F-actin formation, also decreased neutrophil accumulation in the lungs of septic mice. Taken together, our results show for the first time that both adhesive and mechanical mechanisms mediate neutrophil recruitment in the lung. Considering that Y-27632 decreased both Mac-1 up-regulation and F-actin formation in neutrophils, it may be suggested that Rho-kinase signaling regulates both adhesive and mechanical aspects of pulmonary accumulation of neutrophils in abdominal sepsis

5. Conclusion

Our results show that Rho-kinase signaling plays an important role in septic lung damage. Moreover, these findings demonstrate that Rho-kinase-dependent accumulation of neutrophils in lung is composed of both adhesive (Mac-1) and mechanical (F-actin) components in abdominal sepsis. Taken together, our novel data indicate that targeting the Rho-kinase signaling pathway may be a useful strategy to protect against sepsis-induced lung injury.

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