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ORIGINAL ARTICLE

Rho-kinase signalling regulates CXC chemokine formation and leukocyte recruitment in colonic ischemia–reperfusion

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Abstract
Background and aims Leukocyte recruitment is a key feature in ischemia–reperfusion (I/R)-induced tissue injury. The aim of the present study was to investigate the effect of Rho-kinase inhibition on I/R-provoked leukocyte recruitment in the colon.
Methods C57BL/6 mice were subjected to 30 min of ischemia by clamping of the superior mesenteric artery followed by 120 min of reperfusion. Intraperitoneal pretreatment with the selective Rho-kinase inhibitors fasudil (4–40 mg/kg) and Y-27632 (1–10 mg/kg) was administered prior to induction of colonic I/R. Leukocyte–endothelium interactions were analyzed by intravital fluorescence microscopy. Colonic content of tumour necrosis factor-α (TNF-α) and the CXC chemokines macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) were determined by ELISA. Additionally, colonic activity of myeloperoxidase (MPO), a marker of leukocyte infiltration, and malondialdehyde (MDA), were quantified.
Results Fasudil and Y-27632 pretreatment decreased I/R-induced leukocyte rolling and adhesion by 76% and 96%, respectively. Moreover, Rho-kinase interference reduced formation of TNF-α, MIP-2 and KC by more than 68% in the reperfused colon. Additionally, the reperfusion-provoked increase in the levels of MPO and MDA in the colon decreased after Rho-kinase inhibition by 69% and 42%, respectively.
Conclusions Our data demonstrate that inhibition of Rho-kinase activity decrease I/R-induced leukocyte rolling, adhesion and recruitment in the colon. Moreover, these findings show that Rho-kinase signalling regulates TNF-α and CXC chemokine formation as well as lipid peroxidation in the reperfused colon. Thus, targeting Rho-kinase signalling may be a useful strategy in order to protect against pathological inflammation in the colon.

Keywords Adhesion · Colon · Ischemia · Rho-kinase · Microcirculation

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Introduction

The entry of leukocytes into sites of tissue injury requires leukocyte recognition of specific adhesion sites on activated endothelial cells in order to exit the circulation [1]. Ischemia–reperfusion (I/R)-induced extravascular recruitment of leukocytes is a multistep process comprising leukocyte rolling, adhesion and transmigration [2]. In general, it has become increasingly evident that I/R-provoked leukocyte rolling is regulated by P-selectin and its ligand P-selectin glycoprotein ligand-1 [3] and subsequent firm leukocyte adhesion by the integrin superfamily of adhesion molecules [4]. An important regulator of the inflammatory response is tumour necrosis factor-α (TNF-α) which is produced by most cells, including macrophages, mast cells and epithelial cells as part of the response to tissue injury [5-8]. TNF-α is a well-documented stimulator of endothelial cell adhesion molecule expression [9] and has been implicated in I/R-

induced tissue injury. Tissue accumulation of leukocytes is orchestrated by secreted chemokines in inflamed tissues [10]. On a structural basis, chemokines are divided into two major families namely CC chemokines that primarily attract lymphocytes and CXC chemokines that specifically induce neutrophil recruitment. Recent published data suggests that I/R-generated reactive oxygen species (ROS) induce formation of the CXC chemokines macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC), which in turn are powerful initiators of leukocyte rolling and adhesion in the colon [11].

Transmission of extracellular stress signals, such as I/R injury into an intracellular response have been shown to involve small GTP-binding proteins, such as the Rho-family, which in turn act on downstream Rho-kinases [12]. Interestingly, it has been reported that Rho-kinases constitute important mediators not only of vascular contraction but also of cytoskeleton reorganization, cellular morphology, motility and adhesion [13-15]. Rho-kinase activation has been described to be involved in the pathogenesis of septic organ injury and vascular inflammation. Moreover, selective Rho-kinase inhibition with two structurally different substances, fasudil and Y-27632, has indicated beneficial effects in limiting the I/R-induced tissue injury in the heart, brain and kidney [16-18]. However, the potential role of Rho-kinase signalling in I/R-induced formation of pro-inflammatory mediators and leukocyte recruitment in the gastrointestinal tract remains elusive.

Based on the above considerations, the aim of the present study was to investigate the effect of Rho-kinase inhibition on I/R-induced leukocyte recruitment in the colon as well as the underlying mechanisms in terms of cytokine and chemokine formation.

Materials and methods

Animals

All studies were performed on male C57BL/6 mice weighing between 22 and 27 g. Animals had free access to food/tap water on a 12–12 h light–dark cycle and were kept under these conditions for 1 week before used in the experiments. Anaesthesia and analgesia were introduced by a cocktail containing 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight intraperitonally (i.p.). The animals were kept on a heating pad (37°C) during the experiment. The experimental protocol in this study was reviewed and approved by the local ethical committee at Lund University.

Experimental protocol

To investigate the protective role of fasudil and Y-27632 in I/R-induced leukocyte recruitment the animals were pre-treated with fasudil (4–40 mg/kg; Sigma Chemical Co, St Louis, USA) and Y-27632 (1–10 mg/kg; Sigma Chemical Co) 2 h prior to induction of ischemia. The animals were then anaesthetized and the right jugular vein was cannulated with a polyethylene catheter for intravenous (i.v.) administration of fluorescent dyes and additional anaesthesia which in turn was followed by a midline laparatomy. The superior mesenteric artery (SMA) was clamped for 30 min followed by removal of the clamp allowing a 120 min period of colonic reperfusion. Inverted intravital microscopy (IIVM) of the colon where then performed by exteriorizing the colon. Importantly, the SMA is considered the most important vessel for blood supply to the colon in rodents [19]

Inverted intravital fluorescence microscopy

IIVM was performed as previously described [20]. In brief, a 5-min equilibration time was allowed before analysis of leukocyte rolling and adhesion was performed in postcapillary venules (19–34 μm) in the colonic submucosa. Contrast enhancement by i.v. injection of fluorescein isothiocyanate-labelled dextran 150,000 (0.05 ml, 5 mg/ml, Sigma Chemical Co.) and in vivo labelling of leukocytes with rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma Chemical Co.) enabled analysis of leukocyte–endothelium interactions in the microvascular bed. For observations of the microcirculation, we used an inverted Olympus microscope (IX70, Olympus Optical Co. GMBH, Hamburg, Germany) and recorded by a video camera, on tape, for offline analysis of leukocyte–endothelium interactions. Five post capillary venules were evaluated in each animal and leukocyte rolling was measured by counting the number of cells rolling along the endothelial lining during 30 s and is expressed as cells per minute. Leukocyte adhesion was measured by counting the number of cells that adhered and remained stationary during the observation time and is expressed as cells per millimetre. Venular wall shear rate was determined by the Newtonian definition: wall shear rate=8((red blood cell velocity/1.6)/venular diameter) as previously described [21].

Systemic leukocyte count

Blood from the tail vein was mixed with Turks solution in a 1:10 dilution. Leukocyte differentiation was made in a Bürker chamber and registered as either polymorphonuclear (PMNL) or mononuclear leukocytes (MNL).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed by incubating a 3 cm long, well rinsed, segment of the large bowel in 1 ml of DMEM containing 10% foetal calf serum, streptomycin, penicillin and fungizon at 37° in a 24-well plate for 24 h. The medium was subsequently harvested and stored in −20°C until analysis of TNF-α, MIP-2 and KC was performed using double-antibody-specific Quantikine ELISA kits (R&D Systems Europe, Ltd, Abingdon, Oxon, UK) according to the manufacturer's recommendation.

MPO activity assay

A 5-cm long segment of the colon was collected after IIVM, rinsed, weighed, homogenized in 10 ml 0.5% hexadecyltrimethylammonium bromide and stored in −80°C. Next, the sample was freeze-thawed, after which the myeloperoxidase (MPO) activity of the supernatant was assessed. The MPO activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance occurring in the redox reaction of H_2O_2 (460 nm, 25°C). Values are expressed as MPO units per gram of tissue.

MDA assay

Lipid peroxidation in the tissue is used as a well-known indicator of oxidative stress. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) upon decomposition. LPO-586™ is a colorimetric assay designed to quantify MDA. Colonic tissue were collected, rinsed and homogenized in 20 mM phosphate buffer (pH 7.4) and 0.5 M butylated hydroxytolouene. An aliquot (200 ml) of the homogenate was added to a reaction mixture containing 650 μl of *N*-methyl-2-phelondile and150 μl of 12 M HCL and the samples were then incubated on a water bath for 60 min at 45°C. Next, the samples were centrifuged at 15,000×*g* for 10 min and the absorbance of the supernatant was then measured by spectrophotometry at 586 nm.

Quantitative polymerase chain reaction

Colon samples were harvested, rinsed and kept in RNA isolation reagent (RNAlater) at −20°C. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, samples were treated with RNase-free DNase (DNase I; Amersham Pharmacy Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 5 μg of total RNA using Stratascript® First-Strand Synthesis System (Stratagene, AH diagnostics, Stockholm, Sweden) and random hexamer primers. Quantitative PCR analysis was performed in a total volume of 25 μl were each sample contained 125 ng cDNA using Brilliant SYBRgreen quantitative polymerase chain reaction (QPCR) master mix and MX 3000 P QPCR detection system (Stratagene) The thermal cycling conditions entailed 40 cycles: denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. All samples were tested in duplicate and normalized on the basis o their β-actin content. The specific primers were as follows: β-actin (f) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (r) 5′-TCT CCA GGG AGG AAG AGG AT-3′; P-selectin (f) 5′-ACG AGC TGG ACG GAC CCG-3′, P-selectin (r) 5′-GGC TGG CAC TCA AAT TTA CAG-3′; E-selectin (f) 5′-TAC TGT CAG CGG GC TAC AC-3′, E-selectin (r) 5′-GGC ACT TGC AGG TGT AAC TAT TG-3′ and ICAM-1 (f) 5′-GTG ATG CTC AGG TAT CCA TCC A-3′, ICAM-1 (r) 5′-CAC AGT TCT CAA AGC ACA GCG-3′.

Statistics

Statistical evaluations were performed using Kruskal–Wallis non-parametric tests for non-normally distributed variables (Dunn's and Dunnet's post hoc test). The results are presented as mean±SEM; *n* represents the number of animals. Differences were considered to be significant at $P<0.05$.

Results

Rho-kinase inhibition decrease I/R-provoked leukocyte recruitment

Intravital microscopy revealed only occasional rolling and adhering leukocytes in sham-operated animals (Fig. 1a and b). On the other hand, 30 min of ischemia followed by 120 min of reperfusion increased leukocyte rolling and adhesion from 3.4±1.3 cells/min and 2.0±0.4 cells/mm in sham-operated animals up to 54.3±7.0 cells/min and 92.3±18.9 cells/mm, respectively (Fig. 1a and b, $P<0.05$ vs sham, $n=4$–7). i.p. pretreatment with 40 mg/kg of fasudil, 2 h before induction of ischemia, reduced the number of rolling leukocytes to 6.9±0.8 cells/min and the number of adherent leukocytes to 2.8±1.2 cells/mm in the reperfused colon (Fig. 1a and b, $P<0.05$ vs vehicle, $n=4$–7). Interestingly, i.p. pretreatment with the structurally different Rho-kinase inhibitor Y-27632 (10 mg/kg) decreased I/R-induced leukocyte rolling and adhesion down to 12.9±7.3

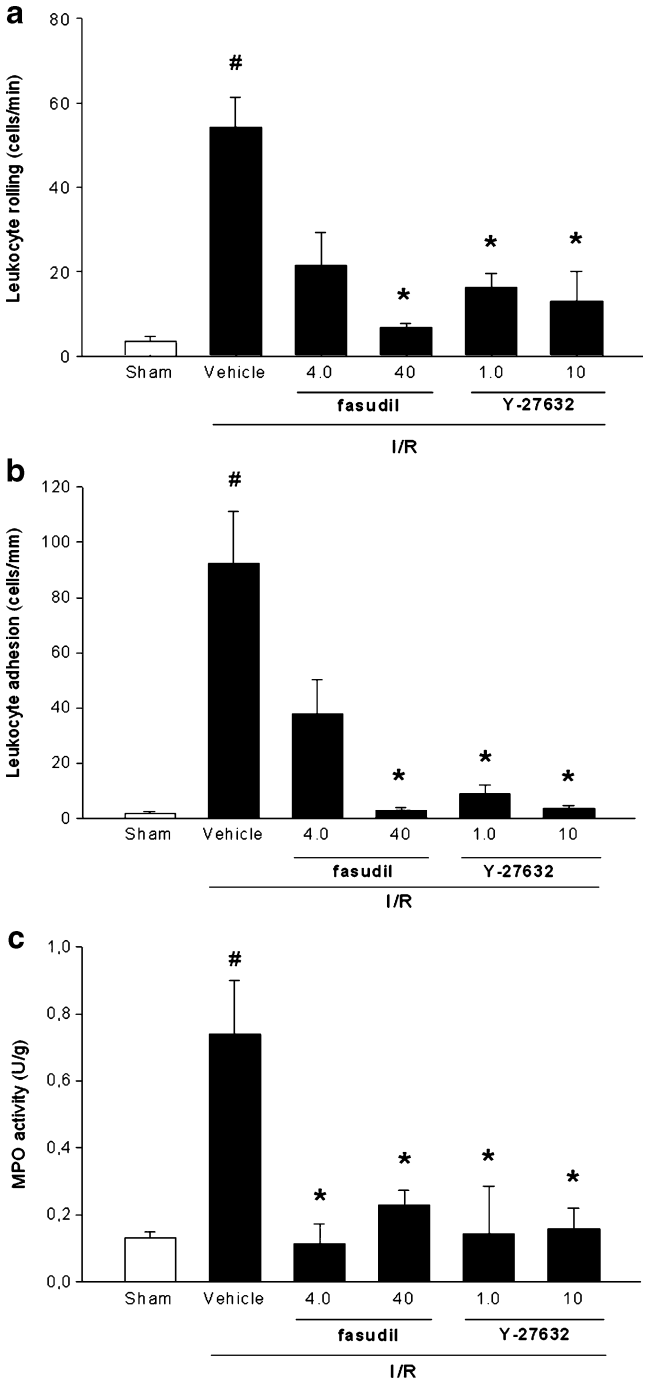

Fig. 1 Leukocyte **a** rolling, **b** adhesion and **c** MPO levels in the colon. Mice were pretreated with fasudil (4–40 mg/kg), Y-27632 (1–10 mg/kg) or PBS (vehicle) i.p. 120 min prior to 30 min of ischemia followed by 120 min of reperfusion. Sham-operated animals received only PBS. Data represents mean±SEM. *$P<0.05$ vs vehicle + I/R and $^{\#}P<0.05$ vs sham (n=4–7)

and 3.6±1.0, respectively (Fig. 1a and b, $P<0.05$ vs vehicle, n=4–7). Thus, inhibition of Rho-kinase signalling reduced I/R-induced leukocyte rolling and adhesion by more than 76% and 96%, respectively.

Rho-kinase regulates colonic CXC chemokine expression

It has previously been observed that colonic I/R-provoked leukocyte rolling and adhesion are mediated by the CXC chemokines MIP-2 and KC [11]. Hence, it was of great interest to investigate whether selective Rho-kinase inhibition of I/R-provoked leukocyte rolling and adhesion was associated with a decreased amount of colonic MIP-2 and KC. In sham-operated animals, levels of MIP-2 and KC were low but detectable. I/R induced a clear-cut increase in the amount of detectable MIP-2 and KC (Fig. 2a and b, $P<0.05$ vs sham, n=5–11). Indeed, i.p. challenge with fasudil and Y-27632 decreased the levels of MIP-2 by 78% (Fig. 2a, $P<0.05$ vs vehicle, n=5–11) and KC by 68% (Fig. 2b, $P<0.05$ vs vehicle, n=5–11) in the colon. Moreover, Rho-kinase inhibition decreased colonic levels of TNF-α by 76% in the reperfused colon (Fig. 2c, $P<0.05$ vs vehicle, n=5–10).

Tissue MPO activity is a marker for neutrophil accumulation and has previously been observed to increase after I/R in the intestine [22]. Hence, after 30 min of ischemia and 120 min of reperfusion the MPO activity was 0.74±0.16 U/g, which was significantly higher than that observed in sham-operated animals (Fig. 1c, $P<0.05$ vs sham, n=5–9). Interestingly, pretreatment with either fasudil (40 mg/kg) and Y-27632 (10 mg/kg) significantly decreased I/R-induced MPO activity in the colon to 0.23±0.04 and 0.16±0.06 U/g, respectively (Fig. 1c, $P<0.05$ vs vehicle, n=5–9). Moreover, MDA content, a marker of lipid peroxidation, increased from 5.2±0.2 to 10.2±0.2 U/g in the colon after I/R (Fig. 3, $P<0.05$ vs sham, n=5–10). Rho-kinase interference decreased reperfusion-provoked increases of colonic MDA in the colon by 42% (Fig. 3, $P<0.05$ vs vehicle, n=5–10). Analysis of the colonic expression of P- and E-selectin and ICAM-1 mRNA was performed by quantitative real-time PCR. Hence, it was found that I/R increased P- and E-selectin and ICAM-1 mRNA levels by 31%, 654% and 66%, respectively (Fig. 4). Fasudil and Y-27632 pretreatment decreased the I/R-generated mRNA production of P- and E-selectin and ICAM-1 amplicons by more than 41%, 81% and 25%, respectively (Fig. 4). Importantly, administration of fasudil or Y-27632 did not change haemodynamic parameters in the colonic microcirculation or systemic leukocyte counts (Tables 1 and 2).

Discussion

The present findings show an important role for Rho-kinase signalling in I/R-provoked leukocyte recruitment in the colon. Hence, this study demonstrates that pretreatment with fasudil and Y-27632 reduce leukocyte rolling, adhesion and extravascular accumulation of leukocytes in the

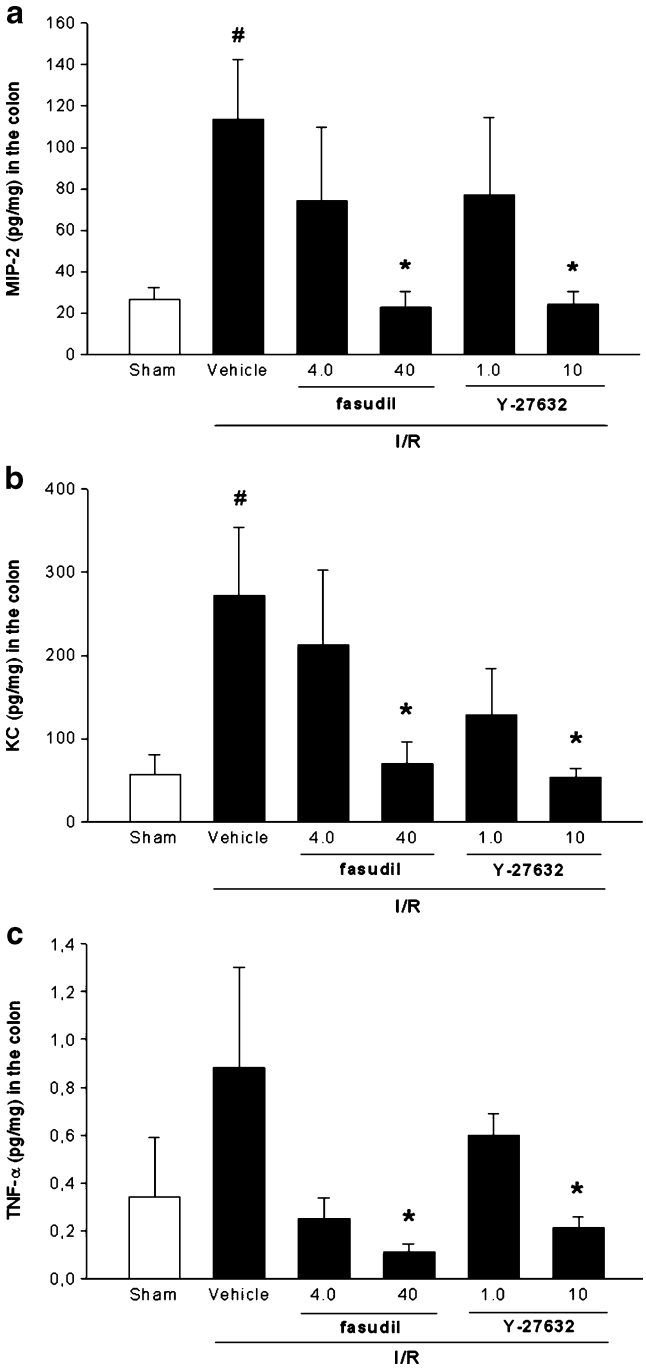

Fig. 2 Colonic production of **a** MIP-2, **b** KC and **c** TNF-α was determined by use of specific ELISA. Mice were challenged with fasudil (4–40 mg/kg), Y-27632 (1–10 mg/kg) or PBS (vehicle) i.p. for 120 min before induction of I/R. PBS-treated animals (sham) served as controls. Data represents mean±SEM. *$P<0.05$ vs vehicle + I/R and $^{\#}P<0.05$ vs sham ($n=5$–11)

reperfused colon. Moreover, these data show that interference with Rho-kinase signalling reduces I/R-provoked upregulation of TNF-α and CXC chemokines (MIP-2 and KC) as well as adhesion molecule expression in the colon. Taken together, our novel data indicate that Rho-kinase

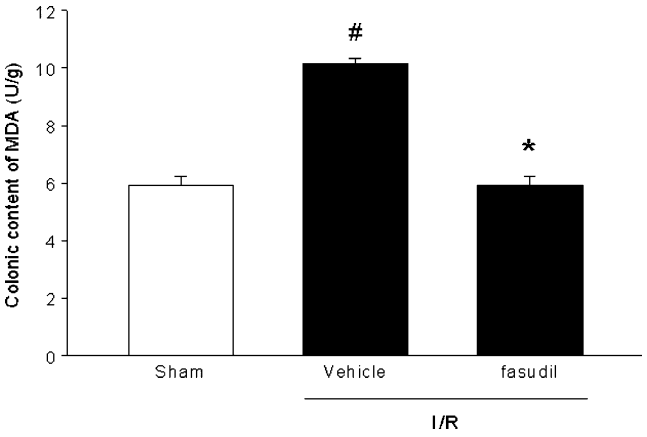

Fig. 3 Content of MDA, a marker for oxidative stress, i.e. lipid peroxidation, as measured by spectrophotometry at 586 nm after PBS (sham) and fasudil (40 mg/kg) pretreatment in the reperfused colon. Data represents mean±SEM. *$P<0.05$ vs I/R and $^{\#}P<0.05$ vs sham ($n=5$–10)

plays an important role in colonic I/R by regulating formation of pro-inflammatory mediators and tissue accumulation of leukocytes. Thus, we suggest that targeting Rho-kinase signalling may be a useful strategy to ameliorate colonic I/R tissue injury.

The Rho/Rho-kinase signalling pathway has generally been considered to regulate cell motility, contractility and cytokenesis via actin stress fibres and myosin phosphatase activity [23-26]. However, emerging data implicates Rho-kinase also in cytokine formation, endothelial nitric oxide production and formation of reactive oxygen species [27-29]. Tissue accumulation of leukocytes is a well-documented component in the inflammatory response

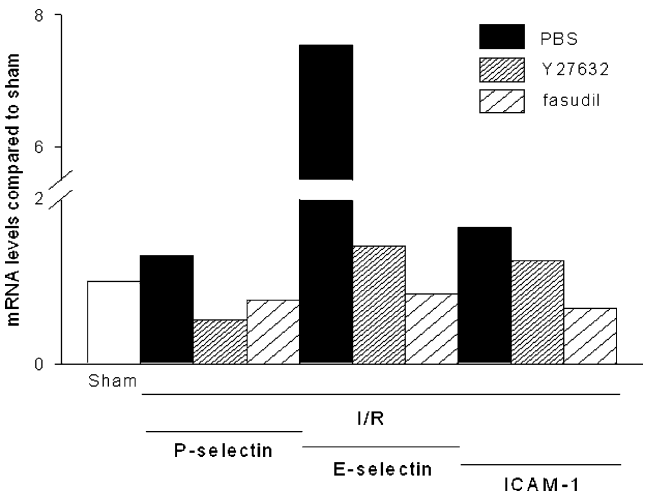

Fig. 4 Endothelial cell expression of P- and E-selectin and ICAM-1 was detected by quantitative RT-PCR after 30 and 120 min of I/R. RNA amplicons were normalized on the basis of β-actin content. Sham-operated animals received PBS whereas mice were challenged with 10 mg/kg Y-27632 and 40 mg/kg fasudil for 2 h before I/R induction and $n=3$

Table 1 Hemodynamic parameters

	Diameter (μm)	Blood cell velocity (mms^{1})	Wall shear rate (s^{-1})
Sham	25.8±0.3	1.4±0.1	144±17
Vehicle + I/R	25.2±0.7	1.1±0.2	152±36
Fasudil 4 mg/kg + I/R	22.6±0.2	1.8±0.2	200±19
Fasudil 40 mg/kg + I/R	22.1±1.4	1.7±0.2	214±36
Y-27632 1 mg/kg + I/R	21.8±1.1	1.2±0.3	151±33
Y-27632 10 mg/kg + I/R	22.9±0.8	1.4±0.3	192±32

Mice underwent I/R for 30/120 min and were pretreated with fasudil (4–40 mg/kg) and Y-27632 (1–10 mg/kg) 2 h prior to induction of ischemia. Sham-operated animals received only PBS. Wall shear rate was calculated from the Newtonian definition: wall shear rate=8 ((red blood cell velocity/1.6) venular diameter). Data are mean±SEM and n=4–7

following I/R and numerous studies have documented that leukocyte recruitment constitutes a rate-limiting step in the inflammatory process. Herein, we found that pretreatment with fasudil and Y-27632, two structurally different selective Rho-kinase inhibitors, markedly attenuated I/R-provoked leukocyte recruitment in the colon. Pretreatment with 40 mg/kg of fasudil and 10 mg/kg of Y-27632 decreased I/R-induced leukocyte rolling and adhesion by more than 76% and 96%, respectively, suggesting that Rho-kinase signalling regulates reperfusion-induced leukocyte–endothelial cell interactions in the colon. These observations extend on a previous study showing that inhibition of Rho-kinase protects against endotoxin-provoked leukocyte adhesion in the liver [30]. Moreover, colonic activity of MPO, a marker for neutrophil accumulation, increased significantly after colonic I/R. Interestingly, Rho-kinase inhibition with fasudil or Y-27632 reduced the colonic levels of MPO by more than 69%. The present findings are, to our knowledge, the first to describe a protective role of Rho-kinase inhibition in I/R-provoked leukocyte recruitment in the colon. Thus, the present data add the colon to the growing list of organs, including liver, heart and brain, which may benefit by interference with Rho-kinase signalling in response to I/R [31-33].

Table 2 Systemic leukocyte differential counts

	PMNL	MNL	Total
Sham	1.0±0.1	3.4±0.2	4.4±0.2
Vehicle + I/R	1.3±0.2	2.6±0.1	3.9±0.1
Fasudil 4 mg/kg + I/R	1.0±0.1	2.7±0.1	3.8±0.1
Fasudil 40 mg/kg + I/R	1.0±0.1	2.9±0.1	4.0±0.1
Y-27632 1 mg/kg + I/R	1.6±0.1	3.0±0.2	4.6±0.3
Y-27632 10 mg/kg + I/R	1.4±0.1	3.3±0.2	4.7±0.2

Mice underwent I/R for 30/120 min and were pretreated with fasudil (4–40 mg/kg) and Y-27632 (1–10 mg/kg) 2 h prior to induction of ischemia. Sham-operated animals received only PBS. Cells were defined as polymorphonuclear or monomorphonuclear and data represents $\times 10^{6}$ cells per milliliter. Data are mean±SEM and n=4–7.

Pro-inflammatory cytokines generated during I/R activate endothelial cells, which in turn express cell surface adhesion molecules supporting interactions between the activated endothelium and leukocytes [34] We found, herein, that Rho-kinase blockade decreased the production of TNF-α, a well-known activator of endothelial cells, by 76% in the reperfused colon. This notion supports the idea that interference with Rho-kinase signalling may indeed exert an anti-inflammatory effect in I/R-generated tissue injury. Moreover, knowing that gut-derived TNF-α in intestinal ischemia-reperfusion injury may cause secondary lung injury [35] it may be speculated that Rho-kinase inhibition may exert protective effect beyond the gastrointestinal tract in colon I/R. Chemokines constitute a large family of small (7–15 kD) structurally related peptides that participate in inflammatory responses through chemo-attraction and activation of leukocytes [36]. CXC chemokines, like MIP-2 and KC, have the capacity to attract and activate predominately neutrophils [10]. Moreover, MIP-2 and KC has been shown to initiate leukocyte rolling and adhesion in the colonic microvasculature [11], which made them particularly interesting in this study. Notably, we observed that challenge with the structurally different Rho-kinase inhibitors fasudil and Y-27632 decreased the I/R-generated colonic expression of MIP-2 and KC by more than 68%, suggesting that Rho-kinase is an important regulator of CXC chemokine generation in I/R in the colon. Thus, this decrease in CXC chemokine production may account for the inhibitory effect of fasudil and Y-27632 on leukocyte recruitment in I/R-provoked tissue injury in the colon. In this context, it is interesting to note that inhibition of ROS formation reduces MIP-2 and KC formation in colonic I/R [11], suggesting that oxidative stress regulates CXC chemokine production. In the present study, we found that inhibition of Rho-kinase activity reduced I/R-induced oxidative stress reflected by diminished lipid peroxidation (MDA formation) in the colon, and this attenuation of oxidative stress may help explain the reduced formation CXC chemokines in the reperfused colon of animals treated with the Rho-kinase inhibitors.

Taken together, our data suggests an important role of Rho-kinase in I/R-induced leukocyte recruitment in the colon. Inhibition of Rho-kinase activity decreased the oxidative stress in the reperfused colon, which was paralleled by a marked reduction in colonic levels of TNF-α and CXC chemokines. Thus, these novel findings indicate that selective targeting of Rho-kinase function could be an effective strategy in preventing pathological inflammatory changes in colonic I/R.

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