

## Role of Rho kinase and actin filament in the increased vascular permeability of skin venules in rats after scalding

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### Abstract

**Objective:** To investigate the role of the Small GTPase Rho and endothelial cytoskeleton in the increased vascular permeability of rat skin after scalding.

**Methods:** Rats were subjected to scalding local ventral skin and a venule was isolated from scalded skin and cannulated by micropipette. The venular permeability was measured with a fluorescence ratio technique and expressed with the permeability coefficient to albumin ( $P_a$ ). The venular F-actin filaments were observed by staining with rhodamine phalloidin and laser confocal scanning microscopy. A specific Rho kinase inhibitor Y-27632 was added into vessel bathing solution or preincubated with vessels to evaluate the role of Rho kinase in regulating of vascular barrier function.

**Results:** Scalding increased  $P_a$  value of skin venule about threefold compared to normal skin venules ( $P < 0.01$ ) and was maintained for 120 min. Inhibition of Rho kinase with Y-27632 (30  $\mu\text{mol/l}$  in low-concentration group; 60  $\mu\text{mol/l}$  in high-concentration group) significantly attenuated the hyperpermeability responses to scalding in a dose dependent fashion. A prominent peripheral actin rim (PAR) existed at the outer area of endothelial cells and apparently delineated the cell-to-cell borders. In the control group, the PARs were arranged smoothly and fairly continuously. However, occasionally PARs did show focal interruption with focal fluorescein isothiocyanate (FITC)-albumin leakage. In the burned group, PARs were less organized and accompanied by a large amount of FITC-albumin leakage. Inhibition of Rho kinase with Y-27632 dramatically reduced  $P_a$  value with recovery of actin filament arrangement in venule after scalding.

**Conclusion:** Burn leads to dermal venular permeability increase with endothelial cytoskeleton depolymerization and disruption. Rho signal transduction pathway is involved in these responses.

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**Keywords:** Rho kinase; Burn; Vascular permeability; Cytoskeleton; Venule

### 1. Introduction

It is well known that burn leads to endothelial barrier dysfunction with increased vascular permeability, the pathogenesis of which is complex, including thermal stimulation, hemodynamics changes, inflammatory mediators and cytokines release. It was shown that the greatest increase in permeability was located in wounded skin and muscle with moderate change in distant organs (liver, spleen, kidney) [1].

Rapid increase of microvascular permeability is associated with the appearance of gaps between microvascular endothelial cells, which is one of the pathways for macromolecule extravasation. Another is the transcellular pathway in which transcellular openings are formed in microvascular endothelium. Some studies suggested that the transcellular

openings may develop from vacuolar channels [2]. However, the mechanism of gap and opening formation has not been fully explained.

During the past several years, the Rho family of small GTPase (Rho, Cdc42, and Rac) has been shown to play a key role in the control of the assembly of the actin-based cytoskeleton and in regulation of cadherin-based intercellular junctions. It has been demonstrated that Rho proteins regulate formation of stress fibers and focal adhesions, Rac proteins regulate formation of lamellipodia and membrane ruffles, and Cdc 42 proteins regulate formation of filopodia [3]. The sequential activation of these three small G proteins by extracellular agonists has been best characterized in quiescent Swiss 3T3 fibroblasts. Agonists such as bradykinin and TNF- $\alpha$  activate Cdc42 proteins in these cells to produce filopodia or microspikes. Activation of Cdc42 proteins leads to localized activation of Rac proteins and formation lamellipodia. Activated Rac proteins lead to weak and delayed activation of Rho proteins that produces stress fibers [4].

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A growing number of studies confirm the role for Rho/Rho kinase signaling transduction pathway in basal endothelial barrier function and in proinflammatory mediators-induced endothelial barrier dysfunction and F-actin reorganization [5–7]. For example thrombin induces a rapid and transient activation of RhoA in human umbilical vein endothelial cells (HUVECs), which is accompanied by an increase in MLC phosphorylation, F-actin stress fibers generation, intracellular small gaps formation and permeability increase in endothelial cell monolayer [8–11]. All of these responses were reduced or abolished by inhibiting RhoA with C3 transferase toxin, a specific Rho inactivator, or microinjecting the isolated Rho-binding domain of Rho kinase, or preincubating with Y-23762 [12] or active MLC phosphatase. Conversely, microinjection of constitutively active V14Rho, constitutively active catalytic domain of Rho kinase, or treatment with the MLC phosphatase inhibitor tautomycin caused contraction. These data suggest that thrombin activates the Rho/Rho kinase pathway to inactivate MLC phosphatase as part of a signaling network that controls MLC phosphorylation/contraction in human endothelial cell.

Several signaling transduction pathways including nitric oxide [13,14], protein kinase C [15], calcium [16] are involved in burn-induced vascular hyperpermeability. However, until now there is no direct evidence that Rho/Rho kinase signaling transduction pathway is involved. Thus, the aim of the present experiments was to test the effects of Rho kinase on actin cytoskeleton and the vascular permeability of venule in rat skin after scalding.

## 2. Materials and methods

### 2.1. General Preparation

Thirty-two male Sprague–Dawley rats, weighing 160–200 g (Laboratory Animal Center of Southern China, China), were anesthetized with a mixed solution of 13.3% ethyl carbamate and 0.5% alphachloralose (0.65 ml/kg) by intramuscular injection. The rats were randomly divided into four groups: control group ( $n = 12$ , 8 for permeability measurement, 4 for cytoskeleton experiment), simple burned group ( $n = 9$ , 6 for permeability, 3 for cytoskeleton), burn + Y1 group (the scalded skin venule treated with Y-27632 30  $\mu\text{mol/l}$ ;  $n = 5$ , for permeability) and burn + Y2 group (the scalded skin venule treated with Y-27632 60  $\mu\text{mol/l}$ ;  $n = 6$ , 3 for permeability, 3 for cytoskeleton). In order to reproduce a burn model, the animals were placed in an appropriate sized template and ventral skin was shaved and scalded with a 90–95 °C hot-water-filled polyethylene membrane bag for 60 s in burn animals. A piece of skin of scalded or unscalded (control) animals area was stripped and placed in 4 °C phosphate-buffered saline (PBS) at pH 7.4. The experiments were approved by the Animal Care Committee of First Military Medical University and performed in adherence to National Institute of Health guidelines.

### 2.2. Solutions and perfusates

An albumin-physiological salt solution (APSS) was used as a bathing solution while the microvessels were being cannulated. It contained the following composition (in mM): 145.0 NaCl, 4.7 KCl, 2.0 CaCl<sub>2</sub>, 1.17 MgSO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.03-(N-morpholino)propanesulfonic acid buffer. After 1% bovine serum albumin was added, the solution was buffered to a pH of 7.40 at 4 °C and then filtered through a Millex-PF 0.8  $\mu\text{m}$  filter unit (Millipore, Bedford, MA). The APSS used to perfuse the microvessels had the same composition as mentioned above but was buffered to a pH of 7.40 at 37 °C.

### 2.3. Isolated and perfused microvessel preparation

The methods for isolating and cannulating skin venules were similar to our previous study [17]. Briefly, a suitable venule (length 1.0–1.2 mm, diameter 60–90  $\mu\text{m}$ , no branch) was isolated from skin in the dissecting chamber containing PBS at 4 °C under a Nikon SMZ-1B stereo dissecting microscope. The isolated vessel was transferred to a cannulating chamber containing APSS at room temperature and cannulated with a micropipette on each end of the venule and secured with 11-0 suture. A third smaller pipette was inserted into the inflow pipette. The vessel was perfused with either APSS through the outer inflow micropipette or APSS containing 0.5% fluorescein isothiocyanate (FITC)-albumin through the inner inflow pipette. Each cannulating micropipette was connected to a reservoir so that the vessel was perfused at a relatively constant intraluminal pressure and flow rate. The bath solution in the chamber was maintained at 37 °C and pH 7.4 throughout the experiments. The vessels were observed and recorded with a Nikon TE300 fluorescence inverted microscope, equipped with a CCD camera system (WAT-902H, Japan) and connected with a computer, equipped with Axon Imaging workbench. The image of the vessel was displayed on a high-resolution video monitor. Diameter of the vessel was measured on-line.

### 2.4. Measurement of venular permeability

The permeability of the vessel was measured with a fluorescence ratio technique. The fluorescent intensity of the interested vessel area was measured and digitized on-line by a computer. In each measurement the isolated venule was first perfused with APSS through the outer inflow pipette to establish baseline intensity. The venular lumen was then rapidly filled with fluorochromes by switching the perfusion to the inner inflow pipette. This produced an initial step increase, followed by a gradual increase, in the intensity of fluorescence. There was a step decrease of intensity when the fluorescently labeled molecules were washed out of the vessel lumen by switching the perfusion back to the outer inflow pipette. The apparent solute permeability coefficient of albumin ( $P_a$ ) was calculated using the equation

$P_a = (1/\Delta I_f) (dI_f/dt)_0 (r/2)$ , where,  $\Delta I_f$  is the initial step increase in fluorescent intensity,  $(dI_f/dt)_0$  is the initial rate of gradual increase in intensity as solutes diffuse out of the vessel into the extravascular space, and  $r$  is the venular radius.

### 2.5. Experimental protocol

In each experiment the cannulated venule was perfused at a constant perfusion pressure gradient of 20 cmH<sub>2</sub>O. This approach produced an approximate intraluminal pressure of 10 cmH<sub>2</sub>O and a flow velocity of 7 mm/s. The preparation was equilibrated for 30–40 min after cannulation, and the measurements were conducted under a temperature of 37 °C and a pH of 7.35–7.45. In each vessel, limited interventions were applied. The preparations were washed three times and allowed to equilibrate for 10–15 min between interventions to ensure that the function of the venular endothelium was not significantly altered with time. For each experimental basal condition  $P_a$  was measured three times and the average value was reported.

### 2.6. Observation by scanning electron microscopy

The isolated vessels were cut open along the vertical section and exposed luminal surface, followed by rinsing twice with PBS and fixed with 2.5% glutaraldehyde in PBS at pH 7.4 for 12 h. They were rinsed with PBS three times again and postfixed with 1% osmium tetroxide in PBS at pH 7.4 for 1 h. The samples were dehydrated in a gradient acetone series, dried with carbon dioxide critical point drying method, and sputter coated with 10 nm of gold. The samples were examined with a scanning electron microscopy (HITACHIS 450, Japan).

### 2.7. Observation of actin cytoskeleton

In order to visualize simultaneously filamentous (F)-actin and leakage of albumin, we used FITC to label albumin, and rhodamine phalloidin to label F-actin in the same isolated venule. The isolated vessel was cannulated at two ends and perfused with APSS for 30 min. In burn + Y group, the vessel was first incubated with Y-27632 60  $\mu\text{mol/l}$  for 40 min. All of preparation was then perfused for 10 min with 0.5% FITC-albumin, followed by fixation with freshly prepared fixative (3.7% formaldehyde in APSS for 30 min). The temperature of bath solution was maintained at 37 °C in above procedure. The preparation was then perfused with a cocktail of 3.7% formaldehyde, [containing: 0.1% Triton X-100, rhodamine phalloidin 2 U/ml (molecular probes, Eugene), and 3.7% formaldehyde in APSS], and the bath solution of vessel was 3.7% formaldehyde for 45 min at 40 °C to stain endothelial F-actin fibers. Subsequently, 3.7% formaldehyde was used as perfusion solution at 37 °C for 30 min again. Finally, the preparation was washed with PBS.

The preparation was mounted on a glass slide and observed with a laser confocal scanning microscope (Leica

SP2, Germany). Magnification of 800–1600 $\times$  was believed to be optimal. An area of interest was identified, and a series of images was collected at different focus levels (step size less than 1  $\mu\text{m}$ ) using the  $\lambda$  568 nm (for rhodamine phalloidin) and the 488 nm (for FITC-albumin).

### 2.8. Statistical analysis

The results are expressed as means  $\pm$  S.D. Student's *t*-test and one-way ANOVA were performed by using SPSS software package. *P*-value less than 0.05 was considered significant.

## 3. Results

### 3.1. Effect of scald on permeability of skin venule

The permeability coefficient to albumin ( $P_a$ ) was measured in venules of similar diameters,  $85.2 \pm 8.3 \mu\text{m}$  and  $83.4 \pm 9.7 \mu\text{m}$  in the simple burned group and control group respectively. The value of  $P_a$  was  $0.62 \pm 0.20$  in scalded skin venule ( $n = 6$ ) and  $0.21 \pm 0.07$  in control skin venule ( $n = 8$ ), which indicated that the venule permeability of scalded skin was increased to threefold relative to normal ( $P < 0.01$ ). To examine whether  $P_a$  was stable during the experiment, the permeability of some venules was continuously recorded for 120 min without any treatment. Fig. 1 shows a typical time course of  $P_a$  changes during the experiments, indicating no significant alteration of  $P_a$  within 120 min of observation.

### 3.2. Effect of Rho kinase inhibitor on burned skin venular hyperpermeability

After measuring  $P_a$ , the burned skin venule was exposed to Y-27632, a specific Rho kinase inhibitor, the change of  $P_a$  was measured. After 30  $\mu\text{mol/l}$  of Y-27632 action, the  $P_a$  value reduced gradually within 10–15 min (Fig. 2).

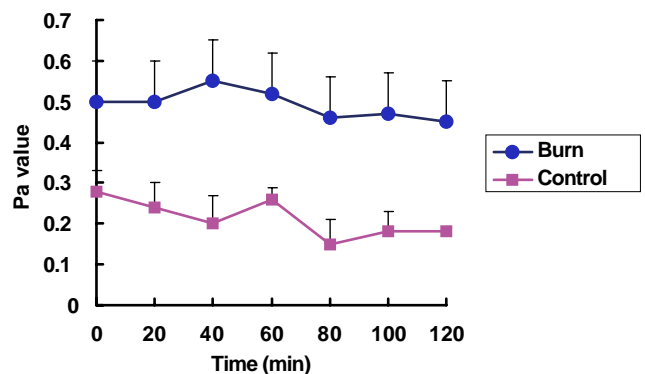


Fig. 1. Time course of apparent permeability coefficient of venule within 120 min. Simple Burn group,  $n = 5$  (one vessel per animal), venular diameter is  $81.4 \pm 7.33 \mu\text{m}$ . Control group,  $n = 4$  (one vessel per animal), venular diameter is  $81.0 \pm 3.5 \mu\text{m}$ .

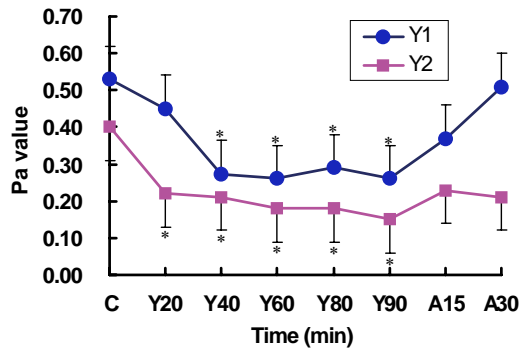


Fig. 2. Effects of Y-27632 on venular permeability in scalded rat skin. Y1 group,  $n = 5$  (one vessel per animal), venular diameter is  $77.0 \pm 4.33 \mu\text{m}$ . Y2 group,  $n = 3$  (one vessel per animal), venular diameter is  $72.0 \pm 5.56 \mu\text{m}$ . (\*)  $P < 0.05$ , compared to no inhibitor.

30 min later  $P_a$  was decreased by 47.8% of basal value ( $P < 0.05$ ), and 90 min later by 50.7%, ( $P < 0.01$ ) in five venules (diameter  $77.4 \pm 4.33 \mu\text{m}$ ).  $P_a$  value was gradually recovered by washing and changing to APSS without inhibitor. Furthermore, Y-27632 decreases post-burn venular permeability in a dose dependent fashion. After application of  $60 \mu\text{mol/l}$  Y-27632, the effect of reducing  $P_a$  was significant at 20 min and maintained for 90 min, which did not reverse by changing to APSS without inhibitor.

### 3.3. Changes of venular endothelial actin cytoskeleton in burns

SEM showed that endothelial cells (ECs) are arranged along the vessel longitudinal axis with fusiform shape and the diameter of each cell is about  $6.5\text{--}10 \mu\text{m}$  (Fig. 3).

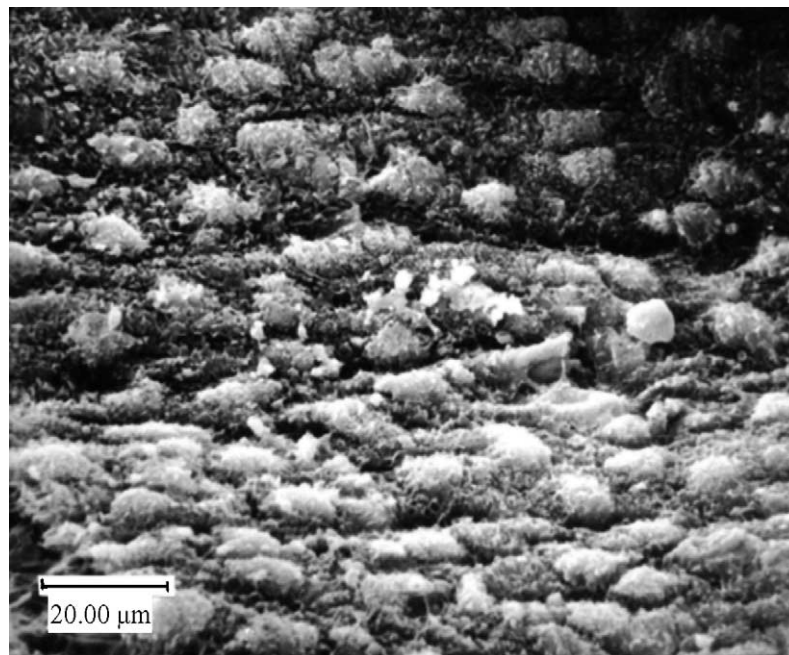


Fig. 3. Scanning electron micrograph of venular endothelium in rat skin.

In control group, F-actins appeared to be present as peripheral fibers at the outer area of the cell and near the cell–cell junctions, which we have called peripheral actin rim (PAR, see arrow in Fig. 4). The PARs were arranged regularly and almost continuously along cell junctions. The width is about  $6\text{--}15 \mu\text{m}$ , and its length is  $10\text{--}40 \mu\text{m}$ , which is near the width and the length of endothelial cells measured by SEM (Fig. 4). Occasional breaks in the PAR were observed where accompanied by a little of FITC-albumin leakage (not shown). In order to examine the reliability of the method, a venule was prepared for observing actin cytoskeleton broken and FITC-albumin leakage after its branch was clamped to injury. Fig. 5 shows the image, in which some actin fibers were broken and disrupted PARs associated with a large of FITC-albumin leakage.

In the simple burned group, it was shown that the PAR depolymerized, regular net construction disappeared, only disrupted F-actin knots remained and a large amount of FITC-albumin leaked out (Fig. 6).

In burned + Y2 group, 40 min after treatment of Y-27632 ( $60 \mu\text{mol/l}$ ,  $37^\circ\text{C}$ ), PARs were seen again in the venule with occasional disruption and only a little of FITC-albumin leaked out, which was similar to it in control group (Fig. 7).

## 4. Discussion

In this study, a method is developed for simultaneously detecting the change of endothelial actin cytoskeleton and albumin extravasation in the same isolated intact venules. In some other studies, burn-serum or burn-neutrophil was added to the normal endothelial cell monolayer derived

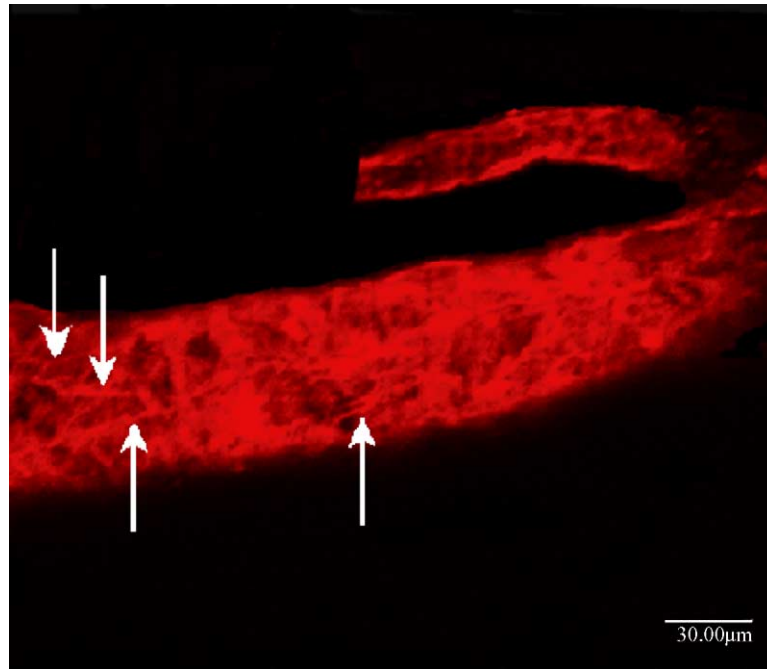


Fig. 4. Confocal Image of rhodamine-phalloidin stained preparation. Note endothelial peripheral actin rim—PAR (arrow) in normal venule.

from human umbilical vein endothelial cell or others [20]. An advantage of our technique is that a real burned skin venule can be obtained for studying the mechanism of hyperpermeability in burns and  $P_a$  value is detected to confirm this enhancement. What happens to the cytoskeleton can be

observed and the Rho A effect in the venule with rhodamine phalloidin—a fluorescent dye specific for actin filament investigated. The present study shows that venular endothelial F-actin is a prominent peripheral actin rim at the outer area of ECs, which may present in a delineate cell-to-cell

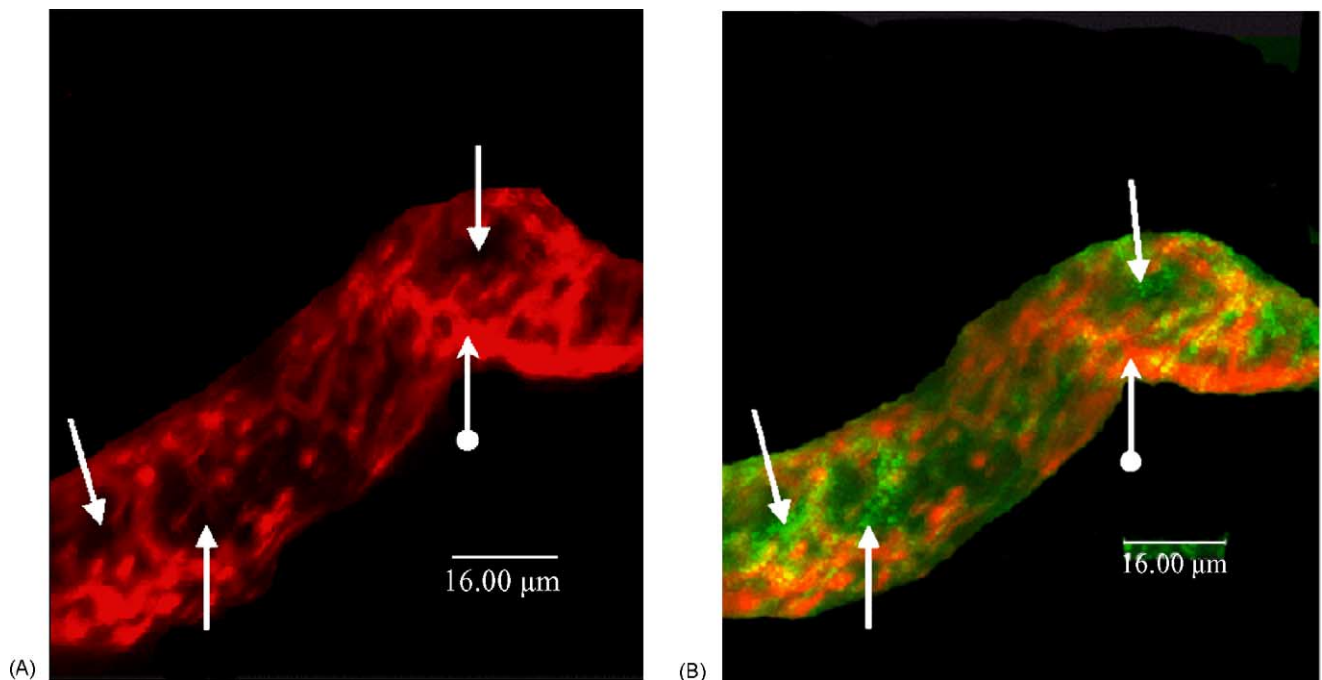


Fig. 5. Image of venular endothelial actin cytoskeleton in control rat skin (branch end was clamped). (A) Rhodamine-phalloidin stained preparation. Note endothelial peripheral actin rim (PAR) in venule (arrow with a circle at the end). Also note disrupted PAR (arrows with no circle at the end) with cave-like appearance. (B) combined image of FITC-albumin and rhodamine-phalloidin staining. Note disrupted endothelial actin fibers associated with FITC-albumin leakage in cave-like place.

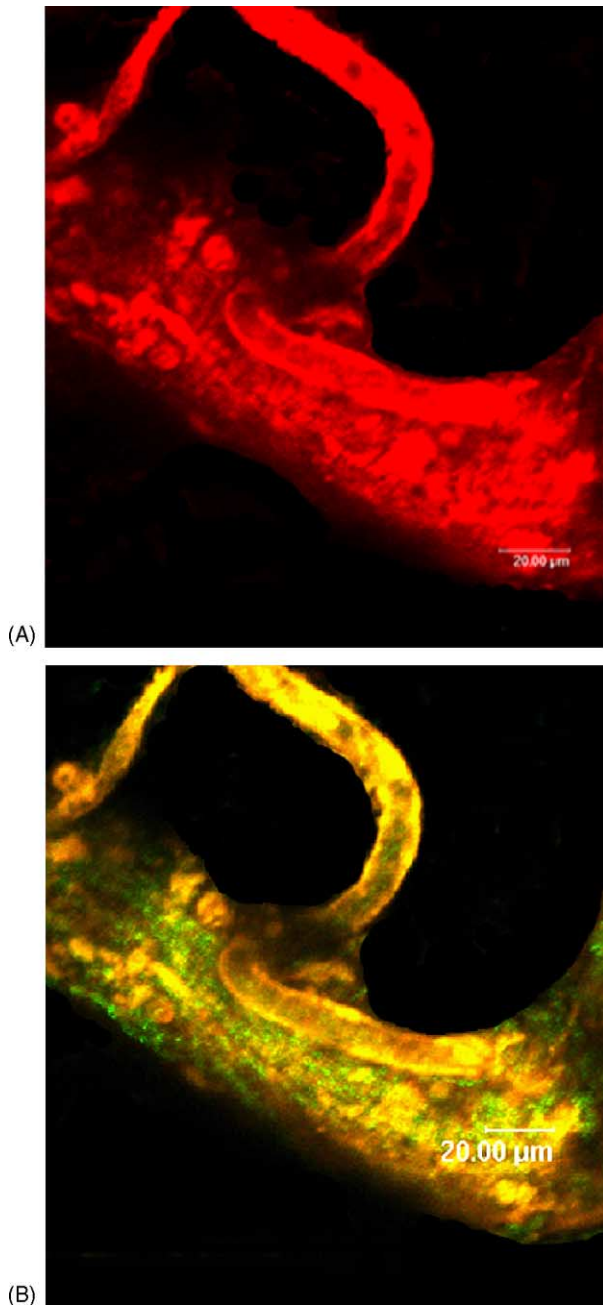


Fig. 6. Confocal image of venular endothelial actin cytoskeleton in scalded skin. (A) Rhodamine-phalloidin stained preparation. Note endothelial peripheral actin rim (PAR) in venule interrupted, net construction disappeared and disrupted F-actin knots remained. (B) Combined image of FITC-albumin and rhodamine-phalloidin staining. Note a large of FITC-albumin leakage disseminated in venule.

borders. In control group, the PARs were smooth and fairly continuous. If we superposed the multilayer pictures obtained from different depths by confocal microscopy, the filament structure was seen to be intercrossed to form a network in appearance. These results are consistent with the actin cytoskeleton of microvessel in rat mesentery and intestine in situ [18,19] and in HUVECs monolayer in which

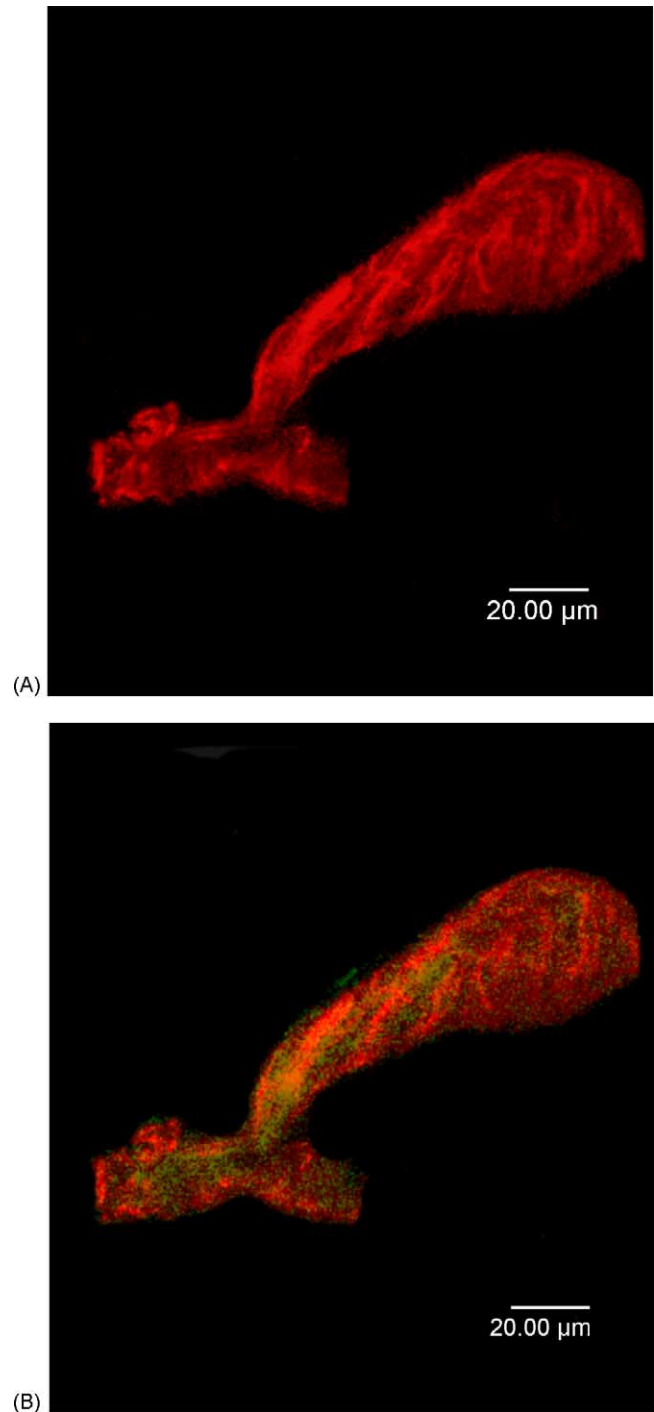


Fig. 7. Effect of Y-27632 on venular endothelial actin cytoskeleton in scalded skin. (A) Rhodamine-phalloidin stained preparation. Note a decrease in PAR disruption. (B) Combined FITC-albumin and rhodamine-phalloidin stained preparation. Note reduced FITC-albumin leakage.

F-actin staining of low intensity was concentrated in the cell periphery area where adjacent cells touched each other [7]. Meanwhile, the diameter of PAR is about 6–15 μm, which is also consistent with the diameter of ECs by SEM. Furthermore, interruptions in the PARs were accompanied by

FITC-albumin leakage. These results indicate that the PAR pattern is cytoskeletons collected from adjacent endothelial cells of venule.

In the present study, we have demonstrated that scald causes a dramatic increase in venular permeability and F-actin depolymerization or disruption in venular endothelium, which was accompanied with a large amount of FITC-albumin leakage. These results are consistent with other reports. It was reported that exposure of human umbilical vein endothelial cells to TNF- $\alpha$  plus burn-neutrophil resulted in rounding and shrinkage of the ECs, with a decrease in actin microfilament number and length. These changes were temporally associated with appearance of intercellular spaces in the monolayer and enhance movement of labeled albumin across the monolayer [20].

It has been shown in this study that Rho/Rho kinase signaling transduction pathway is involved in burn-induced venular hyperpermeability and cytoskeletal changes. Exposure of Y-27632, a specific Rho kinase inhibitor, reduced burn-induced hyperpermeability and preincubation of Y-27632 reversed F-actin depolymerization with decreased FITC-albumin leakage in scalded skin venules of rats. These data suggest that the activating Rho kinase promotes burn-induced vessel hyperpermeability and F-actin depolymerization in rat skin venules. The mechanism of Rho activation in burns remains to be determined. One possibility is that a complex factors including proinflammatory mediators and cytokines release activate Rho protein via G protein coupling receptor on cell membrane, followed by activation of Rho kinase. Activated Rho kinase can phosphorylate MLC phosphatase with inhibition of its activity. Because the phosphatase is inactivated, the level of MLC phosphorylation and myosin activity increases. In addition, Rho kinase directly activates myosin by phosphorylating the regulatory light chain. Activation of myosin causes ATP release and leads to actin–myosin interaction with cell contraction, intercellular gaps formation, and vessel permeability enhancement. Inhibition of Rho kinase with Y-27632 blocked MLC phosphorylation and subsequent cellular contraction with partial recovery of microvascular permeability and actin cytoskeleton distribution.

In conclusion, this study suggests a new method for detecting endothelial actin cytoskeleton in intact venule in vitro and the results show that Rho kinase is involved in burn-induced venular hyperpermeability and cytoskeletal changes. The significance of Rho signal transduction pathway in burn injury is to reveal a new route in the pathogenesis of burn injury. Controlling the Rho signal transduction pathway and maintaining its normal function may help to relieve burn injury. These preliminary observations will lead to a better understanding of burn injury and to increase therapeutic options in the future. Meanwhile, they also provide a basis for further studies in Rho signal transduction pathway.

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