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Research Paper

Involvement of YC-1 in extracellular signal-regulated kinase action in rat cremasteric muscle

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Abstract

Objectives The nitric oxide (NO)–soluble guanylate cyclase (sGC) signalling pathway is attributed to the prevention of ischaemia–reperfusion (I/R)-induced leucocyte–endothelium adhesive interactions. YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), a NO-independent sGC activator, has been shown to exert cardiovascular benefits, but its action on leucocyte–endothelium interactions remains unknown. In this study, the direct effect and the underlying mechanism of the anti-adhesive action of YC-1 have been examined in cremasteric microcirculation.

Methods Rat cremaster muscle was subjected to 4 h pudic-epigastric artery ischaemia followed by 2 h reperfusion and intravital microscopy was used to observe leucocyte–endothelium interaction and to quantify functional capillaries in rat cremaster muscle flaps.

Key findings The values for leucocyte rolling, adhering and transmigrating were 5.5-, 6.9- and 8.8-fold greater, respectively, in I/R than in sham-control animals. YC-1 treatment rescued functional capillary density and reduced leucocyte rolling, adhering and transmigrating in I/R injured cremaster muscles to levels observed in sham-controls. Interestingly, these effects were completely blocked by the MEK (extracellular signal-regulated kinase (ERK) kinase) inhibitor (PD98059) but not by sGC or protein kinase C inhibitors. Cotreatment of PD98059 with YC-1 caused a 3.3-, 7.5- and 8.3-fold increase in the values for leucocyte rolling, adhering and transmigrating, respectively, in postcapillary venules of I/R-injured cremaster muscle.

Conclusions This study has indicated that the anti-adhesive and functional capillary density rescue properties of YC-1 were mediated predominantly by the activation of ERK but not sGC, although YC-1 was identified to be a sGC activator. A better understanding of the action of YC-1 on the microvasculature may help shed light on its therapeutic potential for cardiovascular disease.

Keywords ERK; ischaemia/reperfusion injury; leucocyte–endothelium adhesive interaction; soluble guanylate cyclase; YC-1

Introduction

The re-establishment of blood flow after ischaemia adds paradoxically to the damage caused by prolonged ischaemia. This phenomenon is known as ischaemia–reperfusion (I/R) injury, characterized by perfusion disorders and interstitial oedema resulting from capillary constriction and increased permeability.[1,2] Subsequently, the recruitment and transmigration of leucocytes can compromise the integrity of the microvasculature by plugging the small vessels, while vasoconstriction and cellular and interstitial oedema can result in occlusion of capillaries.[3–5] The strategies for prevention of microvascular dysfunction, and therapeutic options that promote recovery of the dysfunctional microvasculature are important to consider when faced with patients with cardiovascular disease.[6,7]

Nitric oxide (NO) is a key signalling molecule that is involved in the regulation of a variety of biological and physiological processes in mammals. Vast experimental and clinical evidence indicates that reduced bioavailability and/or responsiveness to endogenously produced NO contributes to the development of cardiovascular disease. NO is thought to

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play a protecting as well as damaging role in I/R injury.[8–10] It protects tissues from I/R injury through regulation of the vascular tone, inhibition of thrombocyte aggregation and adhesion, inhibition of leucocyte adhesion to endothelium, free radical scavenging, maintaining normal vascular permeability, inhibition of smooth muscle proliferation, strengthening of the immune system and stimulation of endothelium cell regeneration.[11–15] Other studies, however, have reported that NO administration can be detrimental to reperfused organs.[16,17] Additionally, exogenous NO could bind with reactive oxygen species to form peroxynitrite, which results in endothelial dysfunction.[9,18] Therefore, the use of NO as a therapeutic tool for treating I/R injury is an issue currently under debate.[10,14]

NO binds to a variety of haemoproteins, forming a nitrosyl-iron complex that is required for guanylate cyclase activation and cognate cGMP formation. Subsequently, the function of this enzyme activates a cGMP-dependent protein kinase, protein kinase G (PKG), which regulates a plethora of cellular activity, including vascular tone, cell survival, endothelial permeability, and vascular homeostasis and proliferation.[19,20] Based principally on its protective effects in cardiac and vascular tissues, much emphasis has been placed on identifying upstream pharmacological activators or stimulators of the soluble guanylate cyclase (sGC)–cyclic GMP system. YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), originally characterized as a potent sGC activator in platelets, is capable of exerting multifunctional and broad-ranging effects in the cardiovascular and haematological systems.[21–23] Although YC-1 was first introduced to increase NO–sGC activity and cGMP level on platelets, reponses mediated via a cGMP-independent pathway have also been reported.[21,22,24] In addition, it has been demonstrated that YC-1 activated PKG through an upstream sGC–cGMP pathway to elicit protein kinase C (PKC)–α activation, which in turn, initiated mitogen-activated protein kinase (p44/42 MAPK) activation, and finally induced cyclo-oxygenase 2 (COX-2) expression.[25] In this study, YC-1 was used to act as a microvascular protector of I/R injury and the intracellular signalling pathway by which YC-1–elicited protection in I/R-injured rat cremaster muscle was studied. Specific inhibitors were used to study the downstream signalling pathways and potential pathways that are involved in the benefit of YC-1.

Materials and Methods

Animals

Male Sprague–Dawley rats were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed in an animal room with a constant temperature of 22 ± 1°C and a fixed 12 h light–dark cycle. All animals were handled and housed according to the guidelines and manual of the Committee of the Care of Laboratory Animals of Chang Gung University.

Cremaster muscle preparation

Sprague–Dawley rats (100–130 g) were anaesthetized with inactin (Sigma, St. Louis, MO, USA;100 mg/kg i.p.) and positioned supinely. The cremaster muscles were prepared for intravital microscopy following methods described in previous studies.[13] In brief, surgical exposure of the cremaster muscle began with an incision from the anterior iliac spine to the tip of the scrotum, exposing the inguinal ligament (the most caudal part of the anterior abdominal wall) and the cremaster muscle. The cremaster muscle was then incised along its least vascular sector, and the testicles were removed. With the aid of a stereomicroscope, the cremaster muscle was dissected out as a miniature island flap, attached to the body only by a neurovascular pedicle (pudic-epigastric artery and vein, and genitofemoral nerve). In the I/R group a single microvascular clamp was placed on the pudic-epigastric artery to start the 4-h ischaemia. The 2-h reperfusion was achieved by releasing the clamp applied to the pudic-epigastric artery. The sham-operated control animals underwent all surgical procedures but without their pudic-epigastric artery being clamped. Before and during the ischaemia or reperfusion period, heart rate (HR) and blood pressure (BP) were recorded. In the experimental groups, sham-operated and I/R-injured (4-h pudic-epigastric artery ischaemia followed by 2-h reperfusion) animals were pretreated with 0.1% DMSO (0.1% dimethyl sulfoxide in normal saline, I/R) or YC-1 (Sigma; 0.05, 0.1 or 0.2 mg/kg; dissolved in 0.1% DMSO, i.v.) 10 min before ischaemia (YC-1 (0.05 mg/kg) + I/R, YC-1 (0.1 mg/kg) + I/R, and YC-1 (0.2 mg/kg) + I/R). In some experiments, animals were intravenously pretreated with 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (Sigma; ODQ; 5 mg/kg, an sGC inhibitor), chelerythrine (Sigma; 5 mg/kg, a PKC inhibitor), and PD98059 (Sigma; 1 mg/kg, a mitogen-activated protein kinase kinase inhibitor) for 5 min before treatment with YC-1.

Intravital microscopy

The cremasteric microcirculation was visualized using an intravital microscope (Nikon Measurescope MM-22, Tokyo, Japan) with a 40× objective lens (M plan 40/0.40 SLWD, Nikon, Japan) and a 10× eyepiece. A colour video camera (Sony DXC-750 MD, Tokyo, Japan) was used to project the images onto a calibrated monitor (Sony) and the images were recorded for playback analysis using a videocassette recorder (Sony SVO-9600, Tokyo, Japan). To minimize variability, four to six venules (25–40 μm in diameter) were selected in each trial and the same section of each venule was observed throughout the experiment. The venular diameter, the number of rolling and the number of adherent leucocytes were determined off-line during video playback analysis. Rolling leucocytes were identified as cells moving at a slower velocity than that of the erythrocytes within a given vessel. Leucocyte rolling velocity was determined by measuring the time required for a leucocyte to roll along a 100-μm length of venule. Leucocyte rolling flux was defined as the number of rolling cells moving past a fixed point on the venular wall per minute. Rolling flux was averaged over 2 min. Leucocytes were considered to be adherent to the venular endothelium if they remained stationary for 30 s or longer within a given 100-μm vessel segment. Leucocyte migration was defined as the number of extravascular leucocytes visible per microscopic (40×) video field centred on a postcapillary venule and was determined by averaging the data derived from four to six fields after I/R.

Functional capillary density (flowing capillary density) was measured in three separate regions of the cremaster flap

(proximal, middle, and distal) by counting the number of capillaries (with blood flow) per high power field. The mean capillary density was obtained by averaging the capillary densities from the three regions of each cremaster flap and was used for statistical comparison between different groups.

Statistics

Differences in functional capillary densities and leucocyte counts were analysed statistically. One-way analysis of variance was performed to determine the variables that were likely to show differences between sham-operated, I/R, YC-1 0.05, 0.1 or 0.2 mg/kg + I/R group. These variables were subsequently analysed for significant differences between the groups using Tukey's test. Statistical significance was at $P < 0.05$ and all tests were two-tailed.

Results

YC-1 ameliorated I/R-induced microcirculatory disturbances

Low rates of leucocytes rolling, adhering and transmigrating (1.9 ± 0.65, 1.8 ± 0.37 and 1.0 ± 0.25 leucocytes/100 μm, respectively) were seen in the sham-operated control group. After ischaemia–reperfusion (4-h pudic-epigastric artery ischaemia followed by 2-h reperfusion), a large increase of rolling, adhering and transmigrating leucocytes (10.4 ± 1.44, 12.4 ± 2.02 and 8.8 ± 0.91 cells/100 μm, respectively) were observed in the postcapillary venules of cremaster muscle (Figure 1a). In contrast, animals pretreated with YC-1 (0.1 or 0.2 mg/kg) 10 min before I/R had significantly lower numbers of I/R-induced rolling (1.5 ± 0.52 and 2.2 ± 0.74 cells/100 μm at YC-1 0.1 and 0.2 mg/kg, respectively), adhering (3.1 ± 0.85 and 1.3 ± 0.36 cells/100 μm at YC-1 0.1 and 0.2 mg/kg, respectively) and transmigrating (1.9 ± 0.62 and 1.0 ± 0.26 cells/100 μm at YC-1 0.1 and 0.2 mg/kg, respectively) leucocytes in the postcapillary venules of rat cremaster muscle (Figure 1a).

In the sham-operated control group (baseline), the functional capillary density was 12.8 ± 0.86 per nine fields of view in various experimental animals (Figure 1b). After 4-h ischaemia followed by 2-h reperfusion, I/R-injured rat cremaster muscles exhibited a marked reduction in the number of functional capillaries as compared with the sham-operated control animals (12.8 ± 0.86 vs 9.8 ± 0.83, sham vs I/R, $P < 0.05$). Animals pretreated with YC-1 (0.05, 0.1, or 0.2 mg/kg) before I/R appeared to be protected from the detrimental effects of I/R in the microvasculature. The functional capillary density was 10.2 ± 1.20, 11.9 ± 0.54, and 13.7 ± 0.53 at YC-1 0.05, 0.1, and 0.2 mg/kg, respectively). The number of functional capillaries in the YC-1 (0.2 mg/kg) + I/R group was 39.8% more compared with the I/R group (Figure 1b). Furthermore, before and during the ischaemia or reperfusion period, HR and BP were monitored and recorded. There was no significant difference among the experiment groups.

The microvascular protective effects of YC-1 was antagonized by ERK inhibitor but not by sGC or PKC inhibitors

Under I/R conditions, the anti-adhesive properties of YC-1 in the postcapillary venules only was partially antagonized by

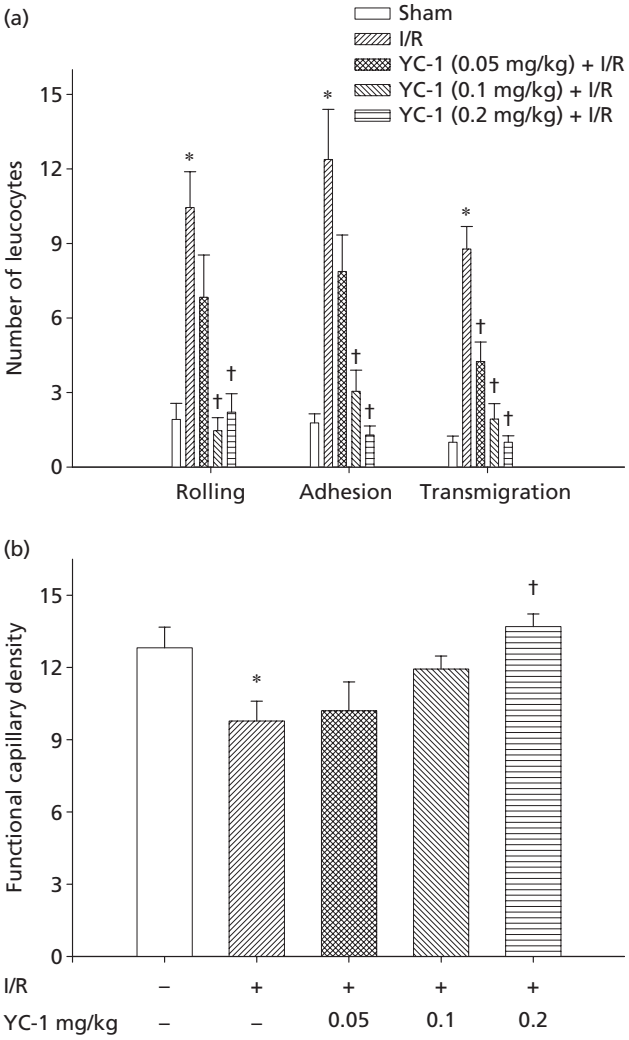

Figure 1 Effects of YC-1 pretreatment and the induction of ischaemia–reperfusion on the number of rolling, adhering and transmigrating leucocytes and the functional capillary density in the microcirculatory system of the rat cremaster muscle. (a) The number of rolling, adhering and transmigrating leucocytes per high-power field (counted over 2 min); (b) the functional capillary density. YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) was used at 0.05, 0.1 and 0.2 mg/kg. I/R, ischaemia–reperfusion. Values are expressed as mean ± SE, $n = 6–8$. *$P < 0.05$ vs sham; †$P < 0.05$ vs I/R.

ODQ (5 mg/kg, a sGC inhibitor) or chelerythrine (5 mg/kg, a PKC inhibitor) in rat cremaster muscle (Figures 2 and 3, respectively). In I/R-injured rat cremaster muscle, the functional capillary density rescue effect of YC-1 was significantly blocked by the PKC inhibitor but not by the sGC inhibitor ODQ. Interestingly, the results showed that YC-1 ameliorated I/R-induced leucocyte rolling, adhering and transmigrating in the postcapillary venules (Figure 4a) as well as salvaging the density of functional capillaries (Figure 4b), but this beneficial effect was completely blocked by cotreatment with PD98059 (1 mg/kg, a MEK (extracellular signal-regulated kinase (ERK) kinase) inhibitor), suggesting the ERK-dependent pathway was involved in the microvascular protective effects of YC-1.

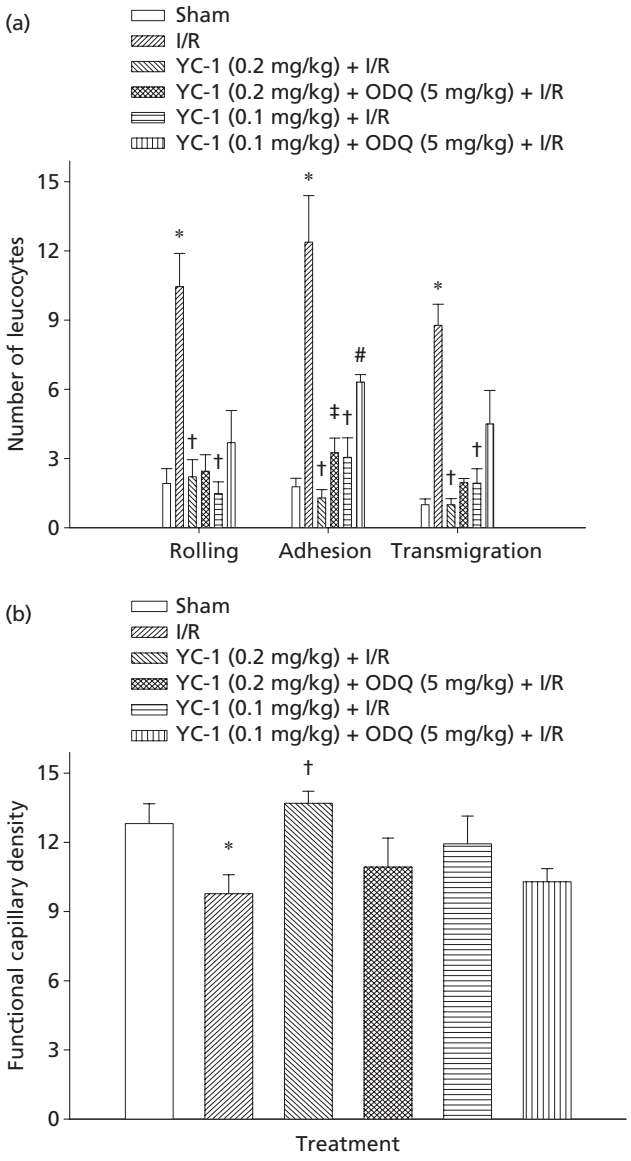

Figure 2 ODQ (sGC inhibitor) partially antagonized the anti-adhesive property of YC-1 in postcapillary venules of ischaemia–reperfusion-injured rat cremaster muscle. (a) The number of rolling, adhering and transmigrating leucocytes per high-power field (counted over 2 min); (b) the functional capillary density in sham-operated control, ischaemia–reperfusion (I/R), YC-1 + I/R and ODQ + YC-1 + I/R groups. ODQ, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole. Values are expressed as mean ± SE, $n = 6$–8. $^{*}P < 0.05$ vs sham; $^{\dagger}P < 0.05$ vs I/R; $^{\ddagger}P < 0.05$ vs YC-1 0.2 mg/kg + I/R; $^{\#}P < 0.05$ vs YC-1 0.1 mg/kg + I/R.

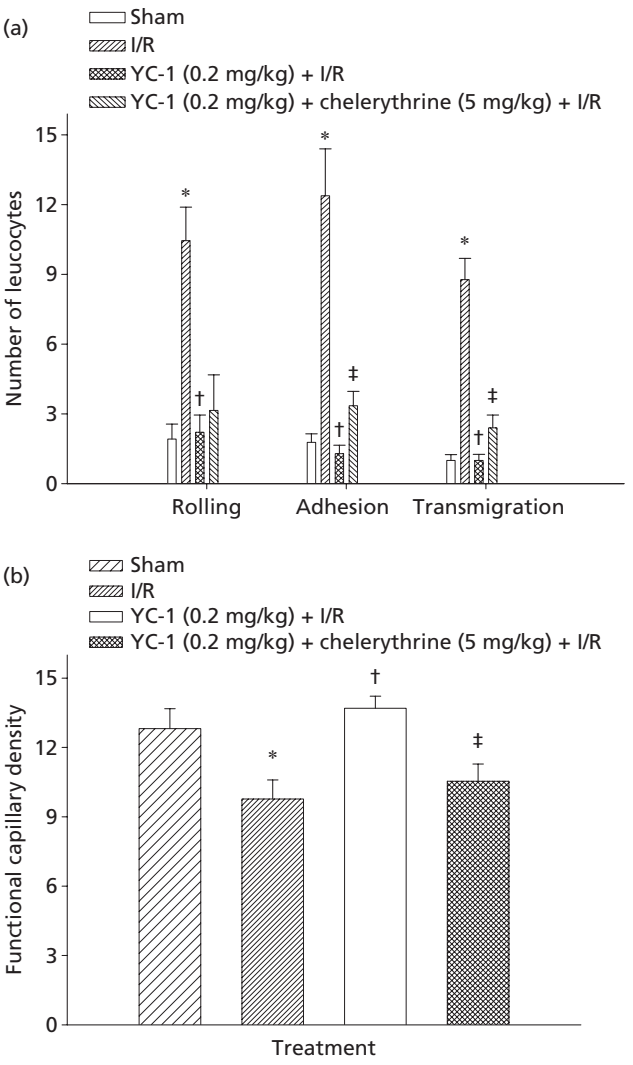

Figure 3 Chelerythrine (PKC inhibitor) partially antagonized the anti-adhesive property in postcapillary venules and significantly blocked functional capillary salvaging of YC-1 in ischaemia–reperfusion-injured rat cremaster muscle. (a) The number of rolling, adhering and transmigrating leucocytes per high-power field (counted over 2 min); (b) the functional capillary density in sham-operated control, ischaemia–reperfusion (I/R), YC-1 + I/R and chelerythrine + YC-1 + I/R groups. YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole. Values are expressed as mean ± SE, $n = 6$–8. $^{*}P < 0.05$ vs sham; $^{\dagger}P < 0.05$ vs I/R; $^{\ddagger}P < 0.05$ vs YC-1 0.2 mg/kg + I/R.

Discussion

Our data demonstrated that YC-1 dose-dependently rescued functional capillary density and reduced leucocyte rolling, adhering and transmigrating in the post-capillary venules of I/R-injured cremaster muscles. The YC-1-induced improvements in microcirculatory disturbances were mediated predominantly by the activation of ERK but not sGC, although YC-1 was identified to be a sGC activator.

The recruitment of immune cells to sites of tissue injury is an important facet of an inflammatory response and is thought to represent a multistage process involving leucocyte rolling, adhesion and migration. NO production by the vascular endothelium exerts an important cytoprotective, antithrombotic influence on the blood vessel wall by preventing the activation and adherence of circulating cells and platelets.[26–28] On the other hand, the pharmacological properties of NO have been exploited for over a century in cardiovascular disease, and to this day, NO donor drugs remain an important feature of cardiovascular medicine[29,30] However, a number of cardiovascular diseases such as

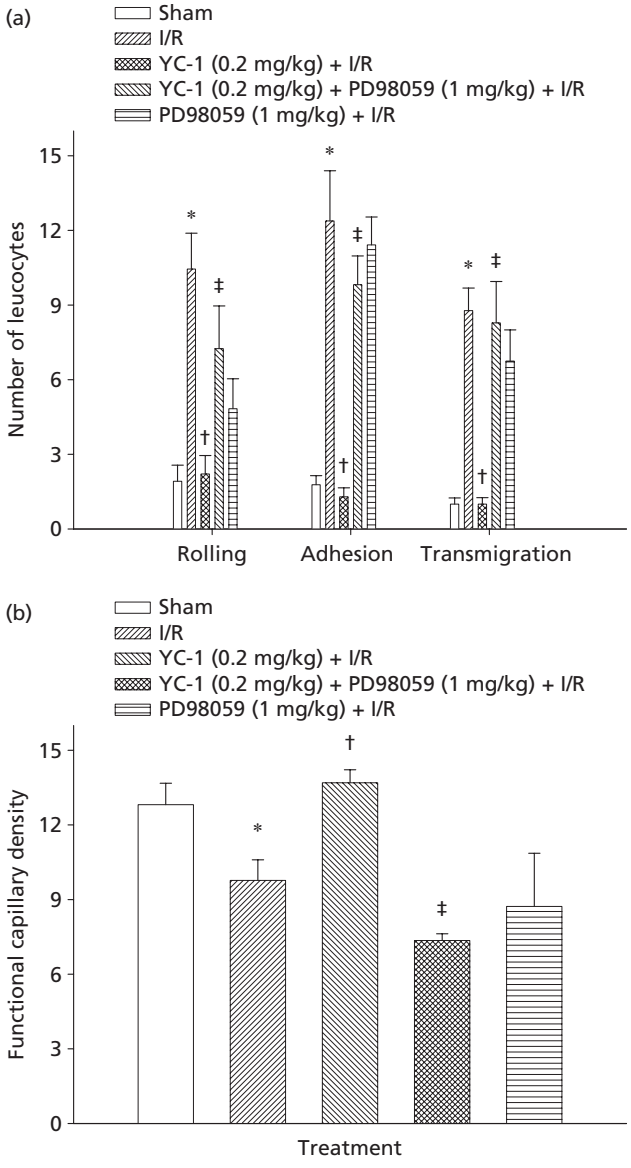

Figure 4 PD98059 (MEK inhibitor) completely blocked the anti-adhesive property and rescued functional capillary perfusion of YC-1 in postcapillary venules in ischaemia–reperfusion-injured rat cremaster muscle. (a) The number of rolling, adhering and transmigrating leucocytes per high-power field (counted over 2 min); (b) the functional capillary density in sham-operated control, ischaemia–reperfusion (I/R), YC-1 + I/R and chelerythrine + YC-1 + I/R groups. YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole. Values are expressed as mean ± SE, $n = 6–8$. *$P < 0.05$ vs sham; †$P < 0.05$ vs I/R; ‡$P < 0.05$ vs YC-1 0.2 mg/kg + I/R.

ischaemic heart disease and hypertension are associated with resistance to NO donor drugs.[31] Furthermore, both inhaled NO and NO donor drugs are associated with additional limitations including many nonspecific (cGMP-independent) effects, and long-term use of NO donor drugs results in the development of nitrate tolerance. These limitations have therefore driven research into drugs that bypass NO and act directly on sGC to restore the sGC–cGMP signalling pathway in cardiovascular disease. The benzylindazole compound YC-1 was the first sGC stimulator to be identified; this compound stimulates sGC activity independently of NO and also acts in synergy with NO to produce anti-aggregatory, antiproliferative and vasodilatory effects.[21–24] YC-1 has beneficial effects in animal models of cardiovascular disease, stimulating a pronounced decrease in mean arterial pressure in a rat model of hypertension, reducing neointima formation following arterial balloon injury in rats and inhibiting of hypoxia-increased blood–brain barrier permeability.[32–34] Whether YC-1 is attributed to prevention of leucocyte–endothelial cell interactions *in vivo* remains unclear. In this study, YC-1 significantly prevented I/R-induced microvascular dysfunction (including reducing leucocyte rolling, adhering and transmigrating and increasing the density of functional capillaries) in the postcapillary venules of rat cremaster muscle.

The mechanism underlying the antiplatelet activity of YC-1 has been well characterized and shown to involve a sGC–acGMP signalling pathway.[21–23] However, YC-1-mediated responses via a cGMP-independent pathway have been reported also.[24,33,34] Hence, the mechanisms by which YC-1 modulated leucocyte–adherence and recruitment in postcapillary venules of cremaster muscle, in terms of the putative second messenger systems, remains to be clarified. In this study, cotreatment with ODQ (a selective inhibitor of NO-sensitive GC) only partially antagonized the preventative effect of YC-1 on leucocyte–endothelial cell adhesive interaction in the postcapillary venules, and incompletely attenuated the rescue effects of YC-1 on functional capillary density. This result indicated the ability of YC-1 to ameliorate leucocyte–endothelial cell interaction in response to I/R injury was not governed by a sGC-dependent mechanism. I/R injury has been shown to activate the prosurvival kinase signalling cascades, PKC and p42/p44 extra-cellular signal-regulated kinases (ERK1/2), both of which have been implicated in cellular survival through their recruitment of anti-apoptotic pathways of protection.[35,36] The pharmacological manipulation and activation of ERK cascades during reperfusion affords an opportunity to attenuate I/R injury, thereby salvaging viable myocardium and limiting infarct size.[37] On the other hand, the ERK1/2 pathway has been shown to regulate vascular endothelial cell permeability, vascular smooth muscle cell contraction and microvascular myogenic tone.[38–41] Recently, it was reported that the ERK signalling pathway was involved in the biological action of YC-1.[25,33,34] The present findings have shown that the YC-1 inhibited microvascular dysfunction in response to I/R injury was entirely blocked by PD98059 (MEK inhibitor) but not chelerythrine (partially antagonized), suggesting that the mechanism underlying ERK-mediated suppression of leucocyte adherence was involved in the protection of YC-1 in in-vivo experimental animals.

Conclusions

Although YC-1 was identified to be a sGC activator, this study has demonstrated that the anti-adhesive and functional capillary density rescue properties of YC-1 were mediated predominantly by the activation of ERK, and not sGC. The study has therefore assigned a previously uncharacterized role to

ERK signalling in regulation of leucocyte–endothelium adhesive interactions and has promoted the concept that YC-1 (a NO-independent sGC activator) is a potent anti-inflammatory agent and may represent a new treatment for inflammatory microvascular disease.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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