A Rho-associated kinase mitigates reperfusion-induced change in the shape of cardiac capillary endothelial cells in situ

Matthew C.P. Glyn\textsuperscript{a,*, b} , John G. Lawrenson\textsuperscript{b} , Barbara J. Ward\textsuperscript{a}

\textsuperscript{a}William Harvey Research Institute, Biomedical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK
\textsuperscript{b}Department of Optometry and Visual Science, City University, London EC1V 0HB, UK

Received 6 May 2002; accepted 26 July 2002

Abstract

Objective: We have previously demonstrated that ischaemia and reperfusion of the myocardium alter capillary dimensions and endothelial cell shape and that these changes are likely to be effected by the actomyosin contractile system in endothelial cells. Rho GTPases are involved in the regulation of cytoskeletal re-organization and in cell contraction. Rho-associated kinase regulates the sensitivity of myosin light chain to Ca\textsuperscript{2+} in smooth muscle but not in cardiac or skeletal muscle myocytes. This study investigated the role of Rho-associated kinase in endothelial cell shape change induced by cardiac ischaemia and reperfusion. The role of Rho proteins in endothelial cell shape change in situ in the myocardial capillary bed has to date not been investigated. Methods: Ischaemia and reperfusion were induced in Langendorff perfused rat hearts at constant flow. Electron microscopy and immunofluorescence studies localized the Rho-associated kinase isotype in capillary endothelial cells. Whole capillary and luminal cross-section areas, luminal and abluminal membrane lengths were measured to monitor changes in cell dimensions. We used a ROCK inhibitor, Y-27632, to investigate the role of this protein in endothelial cell shape change. Results: ROCK1 localized primarily to intracellular membranes in endothelial cells. Morphometric analysis and a study of capillary lumen resin casts demonstrated that inhibition of the activity of this kinase with Y-27632 ablated the change in shape of endothelial cells induced by ischaemia and reperfusion. Conclusion: These results suggest that ROCK1 is involved in cardiac capillary endothelial cell shape change in situ and that targeting the contractile system in this way may be useful in ameliorating reperfusion injury.

Keywords: Capillaries; Contractile function; Electron microscopy; Endothelial function; Ischemia; Reperfusion

1. Introduction

Reperfusion injury in the cardiac microvasculature is associated with a change in the dimensions of the capillary endothelial cells [1]. The localized absence of flow following reperfusion, (no reflow) and reperfusion injury result from several potential factors, catabolite accumulation, free radical release, the effect of cytokines, oedema, and leucocyte plugging eventually causing regional myocyte death [3]. Microvascular reperfusion injury as well as the accompanying changes in endothelial cell dimensions, can be prevented or reduced by agents which target the actomyosin contractile system in cardiac endothelial cells [2,4]. This suggests that the change in endothelial cell dimensions is an active process. Active capillary constriction has been demonstrated in spleen [5] and pancreas [6]. Cultured endothelial cells are able to exert contractile force and cell shape changes in response to mediators, suggesting that endothelial cells are able to contract intrinsically [7,8]. Many vasoactive chemicals induce a reorganization of microfilaments and the cell surface of microfilaments [8,9]. Phalloidin has been demonstrated to prevent the dimensional changes which occur in cardiac capillaries during reperfusion without affecting growth or metabolism [4]. In smooth muscle, Uehata et al. have demonstrated that inhibition of a Rho-associated kinase (ROCK) induces

\textsuperscript{*}Corresponding author. Tel.: +44-207-882-6399; fax: +44-207-9882-6319.
\textit{E-mail address}: m.glyn@qmw.ac.uk (M.C.P. Glyn).
relaxation of rabbit aortic rings by altering the shape of smooth muscle cells [10]. The Rho family of proteins plays a significant part in regulating actin cytoskeleton organization [11]. Regulation of smooth muscle contraction occurs via cytoplasmic Ca\(^{2+}\) and myosin regulatory light chain sensitivity to Ca\(^{2+}\) [12]. The latter is regulated by the activity of smooth muscle myosin phosphatase which itself is regulated by a Rho-associated kinase, a downstream effector of Rho [12–15]. The target kinase does not influence skeletal or striated myocyte myosin sensitivity to calcium. Two isoforms of the Rho-associated kinase exist [11], ROCK1 (ROK\(\beta\), p160ROCK) and ROCKII (ROK\(\alpha\), Rho-kinase). ROCK1 and Rho-kinase are necessary for microfilament reorganisation in fibroblasts and favour stress fibre formation [16]. ROCK1 is involved in bleb formation [17] a process which also occurs in cardiac capillary endothelial cells. Fibroblasts, smooth muscle and endothelial cells share a common origin (mesenchyme) and under certain conditions embryonic endothelial cells differentiate into smooth muscle cells [18]. In addition, the actin (10%) and myosin (6.2%) content of endothelial cells is significantly greater than that for other nonmuscle cells [19] suggesting a contractile role as reported previously [4]. Three blocks from each heart were sectioned and from 4 to 7 images of capillary cross-sections were captured from two non-consecutive sections from each block.

2. Methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.1. Perfusions

Hearts were excised from freshly killed, by cervical dislocation, male Wistar rats, ~250 g in weight. They were prepared, perfused and fixed as described in Glyn and Ward [4]. The perfusate was maintained at a flow rate of 10 ml min\(^{-1}\) g\(^{-1}\) at 37 °C. All experiments began with an equilibration period of 15 min. A transducer measured aortic perfusion pressures constantly. Turning the pump off and on induced ischaemia and reperfusion, respectively. Hearts were then perfused with fixative [4], Y-27632, a gift from Yoshitomi Pharmaceutical Industries (7-25 Koyata 3-Chome Iruma-shi, Saitama 358, Japan), was diluted in the perfusate.

All experiments involving Y-27632 and morphometry lasted 110 min. Four categories of experiment were carried out as follows.

1. Control experiments where hearts were constantly perfused (without Y-27632) for 110 min, then fixed (n=4).
2. Control experiments where hearts were constantly perfused for 15 min without Y-27632 followed by another 95 min with 10\(^{-6}\) M Y-27632 solution and then fixed (n=6).
3. After the 15-min equilibration period hearts were perfused without Y-27632 for 30 min followed by ischaemia for 45 min, reperfusion for 15 min without Y-27632 and then fixed (n=4).
4. The hearts were perfused normally for 15 min with solution without Y-27632 followed by 35 min with Y-27632, subjected to ischaemia for 45 min, reperfused for 15 min with Y-27632 and then fixed (n=6).

2.2. Transmission electron microscopy

Tissue samples were processed for electron microscopy as reported previously [4]. Three blocks from each heart were sectioned and from 4 to 7 images of capillary cross-sections were captured from two non-consecutive sections from each block.

2.3. Resin casts and scanning electron microscopy

Resin casting was performed as described previously [4]. These experiments lasted for 110 min with the exception of hearts used for ischaemic resin casts which were fixed after 95 min. In all experiments, resin was infiltrated into the microvasculature [11] and then processed for scanning electron microscopy. Four categories of experiments (n=4) were carried out.

1. Control hearts were perfused for 110 min and then fixed.
2. An ischaemic period of 45 min was induced following a 50-min period of normal oxygenated perfusion. The ischaemic hearts were then fixed.
3. Ischaemia for 45 min was induced after 50 min of oxygenated perfusion. Hearts were reperfused for a further 15 min before being fixed.
4. The reperfusion experiments described above were repeated with Y-27632 in the perfusion buffer.

2.4. Morphometric measurements

Images were grabbed from the electron microscope and morphometric analysis performed as reported previously [4,20]. Cross-sectional area of capillaries, lumen and endothelial cells, as well as luminal and abluminal perimeters were measured. The final number of capillary images used in morphometric analysis of each group was as
follows: control 129, reperfused 145, control with Y-27632 146 and reperfused with Y-27632 163.

2.5. SDS–PAGE and Western blotting

SDS–PAGE and Western blotting were performed essentially as described previously [21]. Molecular weight markers used were as follows: myosin 204 kDa, β-galactosidase 116 kDa, bovine serum albumin 102 kDa, and ovalbumin 50 kDa.

2.6. Immunofluorescence assay

To confirm the presence of ROCK1 in endothelial cells, immunofluorescence assay was performed on myocardium perfused for 15 min with oxygenated medium. Samples were prepared as previously described [21]. The primary antibodies against β actin and Rho-associated kinase 1 (ROCK1) [22] were from Sigma–Aldrich and Transduction Laboratories (R810), respectively. Images were taken using a Molecular Dynamics CLSM 2000 confocal microscope and processed using Adobe Photoshop version 4.01.

2.7. Immunogold labeling

A pre-embedding staining technique derived from the immunofluorescence assay protocol was used to reduce potential artifactual differences in labeling patterns. This immunogold protocol, described previously [20], was identical to that used for immunofluorescence until incubation with the secondary gold tagged antibody.

2.8. Statistics

Using Excel 5 and XLSTAT.PRO version 4.4, a one-way ANOVA (analysis of variance) with Bonferroni post-hoc tests for unplanned comparisons was carried out using measurements corrected for obliqueness as described by Ward and McCarthy [1]. When $P$ values were less than 0.05, statistical significance was considered reached.

3. Results

3.1. Capillary cross-sections

The typical cross sectional appearance of oxygenated cardiac capillaries perfused for 110 min without Y-27632 is depicted in Fig. 1A. The myocardium was well preserved. The endothelium was of a relatively uniform width and the cytoplasm contained many vesicles and caveolae. The lumen cross sectional area was large compared with that of the narrow capillary walls. Hearts continued to beat with perfusion pressures ranging from 60 to 75 mmHg until fixed. Another set of experiments demonstrated that the addition of Y-27632 to the perfusate did not result in any significant changes to capillary dimensions in comparison with control capillaries (Fig. 1B). Myocyte structure also was unaffected. Control hearts (oxygenated) perfused with Y-27632 similarly continued to beat until fixed.

Typical morphology of ischaemic/reperfused capillaries without Y-27632 is shown in Fig. 1C. Capillary dimensions were altered considerably; the ratio of the luminal cross sectional area to endothelial cell area had decreased. In these capillaries, in which endothelial cells had undergone moderate swelling, average luminal diameter decreased significantly, while capillary diameter changed little. Myocytes showed typical reperfusion damage with disrupted cristae in swollen mitochondria and irregular swollen myofibrils.

The prophylactic effect of Y-27632 is demonstrated by Fig. 1D. Capillary and endothelial cell cross-sections retained proportions typical of control capillaries not subjected to ischaemia and then reperfused. In this last set of experiments, ischaemic capillaries were reperfused with Y-27632. Endothelial morphology was similar to that in control capillaries with dense cytoplasm containing organelles, caveolae and vesicles. The endothelium was of a similar thickness to that seen in control endothelium. Myocyte structure was well preserved and was similar to control hearts. The protective effect of Y-27632 on the myocardium was also demonstrated by the vigorous and regular contractions of reperfused hearts following 45 min of ischaemia.

3.2. SDS–PAGE and Western blotting

The results of SDS–PAGE and Western blotting experiments are shown in Fig. 2. They demonstrate specificity of the antibody against ROCK1 by detection of a single major protein in whole cell homogenates of rat myocardium and kidney (positive control). Using the molecular weight markers as reference, this protein was ~160 kd, the expected size of ROCK1. Faint smaller molecular weight bands in the Western blots suggest some degradation of the kinase may have occurred.

3.3. Immunofluorescence assay

Immunofluorescence assay was carried out using the antibody against ROCK1 and, separately, a second antibody against β actin which preferentially decorated the luminal surface of the capillary endothelial cells (Fig. 3A). ROCK1 was concentrated in clusters along the capillary luminal border, Fig. 3B and C’. A three-dimensional reconstruction of a capillary probed for ROCK1 is depicted in Fig. 3B. In this micrograph the images of six serial sections, each separated by 0.45 μm, have been combined. In some cases clusters were so close together that they
Fig. 1. (A) Typical transverse section of a capillary and myocytes perfused for 110 min with well oxygenated perfusion medium. (B) Transverse section of a capillary, perfused as in (A) with Y-27632. The capillary is thin walled, the myocytes and mitochondria are well preserved. (C) A typical example of an ischaemic/reperfused capillary (without Y-27632) demonstrating a much thicker capillary wall, swollen mitochondria and damaged myocytes. (D) An ischaemic/reperfused capillary exposed to Y-27632. The capillary is similar to those in (A) and (B). Myocytes and mitochondria are well preserved; there is no significant structural damage. Bar=μm.

appeared contiguous, Fig. 3D and E. These micrographs demonstrate that nuclear staining was absent or if present, was sparse. Propidium iodide was used to highlight the endothelial cell nuclei either in a dual fluorescence assay (D and E) or separately (C). It was impossible to determine, by immunofluorescence, whether the kinase occurred predominantly in the cytoplasm, or associated with the endothelial cell membrane.
3.5.1. Endothelial cell area

Endothelial cell area was increased in ischaemic reperfused capillaries from $2.14 \pm 0.07$ in controls to $2.9 \pm 0.17$ ($P<0.05$). This increase was abolished ($P>0.05$) when Y-27632 was included in the perfusate; cell area remained at control values. Controls with, $2.38 \pm 0.09$, and without Y-27632, $2.14 \pm 0.07$, were not significantly different ($P>0.05$).

3.5.2. Whole capillary area

Y-27632 without ischaemia/reperfusion did not affect capillary area significantly; $11.8 \pm 0.5$ and $10.94 \pm 0.57$, respectively ($P>0.05$). There was no significant difference ($P>0.05$) between the controls with Y-27632 and ischaemic/reperfused with Y-27632 $(10.94 \pm 0.57$ compared with $11.44 \pm 0.7$). This is in contrast ($P<0.05$) to the large reduction in capillary area between controls without Y-27632, $11.8 \pm 0.5$, and ischaemic reperfused without Y-27632, $7.39 \pm 0.54$.

3.5.3. Luminal area

The luminal area measurements gave similar results. Y-27632 alone did not reduce the luminal area significantly ($P>0.05$) but the large reduction in luminal area from $9.66 \pm 0.4$ in controls without Y-27632 to $4.48 \pm 0.39$ in ischaemic/reperfused without Y-27632 ($P<0.05$) did not occur in Y-27632 perfused hearts $(8.56 \pm 0.49$ in controls with Y-27632, $9.15 \pm 0.62$ in ischaemic reperfused with Y-27632, $P>0.05$). The luminal area was significantly greater in ischaemic/reperfused capillaries with Y-27632 than without ($P<0.05$).

3.5.4. Perimeters

Y-27632 did not affect capillary or luminal perimeters, significantly, in well-oxygenated controls. Measurements for controls without Y-27632 were $12.8 \pm 0.29$ and $11.75 \pm 0.27$, respectively ($P>0.05$). The averages for controls with the drug were similar: $12.32 \pm 0.32$ and $11.02 \pm 0.3$ ($P>0.05$). The capillary and luminal perimeters of ischaemic/reperfused capillaries, $10.04 \pm 0.38$ and $8.3 \pm 0.38$ respectively, were significantly different from those of all other groups ($P<0.05$) including ischaemic/reperfused with Y-27632 $(12.42 \pm 0.37$ and $11.43 \pm 0.36$).

3.6. Resin casts

Differences between well-oxygenated capillaries and capillaries subjected to ischaemia and reperfusion are shown in typical images of corrosion casts in Fig. 6. In Fig. 6A, branching capillaries are relatively regular and cylindrical, occasionally demonstrating narrowed regions. Capillaries in Fig. 6B were subjected to ischaemia for 45 min. These were significantly more irregular with a rough, angular topography. Unlike well-oxygenated capillaries,
these vessels were severely narrowed at points along their lengths (arrows). The capillaries depicted in Fig. 6C were reperfused following 45 min of ischaemia after exposure to Y-27632 and resembled oxygenated control capillaries. Capillaries reperfused following 45 min of ischaemia under the same conditions as those in Fig. 6C, but without exposure to Y-27632, are shown in Fig. 6D. During resin infiltration, many capillaries were ruptured (arrows) by the reperfusion pressure which was significantly greater than that of perfusion without any ischaemic insult. Capillaries successfully infiltrated with resin, however, cast with characteristics reminiscent of well-oxygenated capillaries: regular topography and cylindrical shape (arrow heads).

4. Discussion

Changes in cell shape in cardiac capillary endothelial cells caused by the activity of agents such as bradykinin, hydrogen peroxide and thrombin result in increased endothelial barrier permeability [23]. The mechanism involves endothelial cell contraction to form intercellular gaps. The role of the actomyosin contractile system in maintaining vascular permeability has been documented [7] and is inferred by the effect of microfilament poisons on endothelial barrier function [24]. We propose that the microfilament system employed in permeability regulation is also involved in changes in endothelial cell shape [1]. In
cardiac capillary endothelial cells, ischaemia alone and reperfusion induce changes in cell shape. We have shown that the microfilament system is reorganised during these changes and that they are sensitive to agents which target actin [4,20]. In this report we show that change induced by reperfusion may also be prevented by targeting the myosin component of the contractile system using an agent specific for a regulator of the contractile process. This regulator, ROCK1, controls the Ca\textsuperscript{2+} sensitivity of myosin by inhibiting myosin phosphatase [13]. Y-27632 inhibits ROCK1 activity [10] resulting in dephosphorylation of myosin regulatory light chain [14]. For any given Ca\textsuperscript{2+} concentration this favours relaxation of myofilaments.

4.1. Period of ischaemia

Although variation in the condition of endothelial cells subjected to ischaemia is considerable, damage sustained
Fig. 5. Morphometric data of endothelial cell dimensions in cardiac capillaries after perfusion, and reperfusion following ischaemia. (A) Endothelial cell, capillary and luminal cross-sectional area measurements demonstrating the protective effect of the kinase inhibitor Y-27632 (Ki). Cell area increased in ischaemic/reperfused capillary endothelial cells. This increase was prevented by Y-27632. Luminal area was least in capillaries reperfused without Y-27632. Y-27632 did not affect cross-sectional cell area in oxygenated endothelial cells but prevented the reduction in capillary area on reperfusion. (B) Perimeter measurements from the same experiments as in (A). Capillary perimeters reduced following reperfusion. Reduction in luminal perimeters in capillaries was prevented with the Y-27632 perfusion buffer. An initial 15 min of oxygenated perfusion was included in all groups. Key: control, perfused without intervention; IR, perfused for 45 min followed by ischaemia for 45 min and reperfusion for 15 min; CKi, perfused without intervention. Y-27632 included after 15 min perfusion; IRKi, perfused for 45 min followed by ischaemia for 45 min and reperfusion for 15 min. Y-27632 included after 15 min perfusion.

by most cardiac endothelial cells during 30 min of ischaemia followed by reperfusion is reversible. If the period of ischaemia is increased to 60 min, damage is not reversed during reperfusion [1]. For morphometric observations, therefore, we chose an ischaemic period of 45 min to generate endothelial cells which could be retrieved from their damaged state as well as irreversibly damaged cells. For immunofluorescence and immunogold labeling studies, we used hearts perfused for 15 min with oxygenated medium.

4.2. Immunofluorescence assay

The antibody against ROCK1 has been used previously to detect the kinase by Western blotting and immunofluorescence assay [22]. Similarly, we have shown that the antibody detected ROCK1 and used it in immunofluorescence and immunogold labeling assays. Although previous studies have suggested a role for ROCK in the regulation of permeability in pulmonary vasculature [25,26], this is the first direct identification of this kinase in cardiac capillary endothelial cells in situ. ROCK activity has been reported in smooth muscle, fibroblasts and cultured endothelial cells [10,22,27] and might be expected in endothelial cells in situ since all three cell types share a common origin and embryonic endothelial cells differentiate into smooth muscle cells in some conditions [18]. Our immunofluorescence assays suggested that ROCK1 was
primarily localized to or closely associated with the luminal membrane and to a lesser degree with the abluminal membrane. This is consistent with a previous report of membrane-associated ROCK1 activity [28] in resting platelets. Taggart et al. [27] demonstrated a similar association of ROCKII to the periphery of stimulated smooth muscle cells close to the cell membrane. Using immunofluorescence assays, they were unable to determine whether ROCKII was bound to the cell membrane or only associated with it. Rho, which binds ROCKII, demonstrates activity only when it is bound to cell membrane [27,28]. We used immunogold labeling with the antibody against ROCK1 to examine, in greater detail, the association of ROCK1 with the cell membrane suggested by immunofluorescence assays.

4.3. Immunogold labeling assays

Immunogold labeling studies confirmed that ROCK1 activity was associated with the luminal membrane but showed that cytoplasm was also implicated. The distribution of this kinase contrasted with that of β actin which was concentrated along the luminal membrane and was intimately associated with it. Labeling with the antibody against ROCK1 suggested that ROCK1 activity was localized to at least three compartments in oxygenated
endothelial cells. Gold beads decorated the luminal membrane most densely adjacent to the nucleus but the abluminal membrane was also decorated and some labeling occurred in the cytoplasm. This kinase was, however, detected at the periphery of the nucleus suggesting an association with the nuclear membrane. Cytoplasmic ROCK1 activity was the greatest between the nucleus and the luminal membrane. No activity was detected in myofibrils. ROCK1 was also localized to cell membranes and to the cytoplasm of pericytes. In smooth muscle cells induced to change shape, Taggart et al. [27] demonstrated translocation of ROCKII from the cytoplasm to the cell periphery, suggesting cell membrane association. In a constant environment, adequately oxygenated Langendorff-perfused cardiac endothelial cells do not change shape significantly [1,29]. Conditions employed in our experiments, however, were not identical to those in vivo and may have been sufficiently different to induce some activation. Alternatively, relaxed endothelial cells may express some membrane-associated ROCK1 activity as do resting platelets [28].

Essler et al. [29] have reported that ROCK plays a significant role in permeability regulation. A moderate change in cell shape is required to regulate inter-endothelial cell gaps. There is, possibly, a similar redistribution of ROCK1 during activation of the endothelial cell microfilament contractile apparatus. This is consistent with the membrane association of other Rho proteins; Rho, which binds ROCKII, is active only when bound to cell membrane [27,28]. We are currently investigating whether ROCK1 distribution is altered during ischaemia and reperfusion. Under these conditions, endothelial cells change shape resulting in changes in capillary morphometry and endothelial permeability. ROCK1 activity near or associated with the nuclear membrane may, however, be related to other functions, for example transcription [30]. Alternatively, the localization of ROCK1 near the nucleus may be involved in maintaining the three-dimensional positioning of the endothelial cell nucleus. In cultured fibroblasts there are two distinct sets of stress fibres; a peripheral set and a set positioned more centrally within the cell [16]. The latter is more sensitive to Rho kinase activity than that of myosin light chain kinase. This may be related to our finding of ROCK1 activity close to the nuclear membrane.

4.4. Morphometry and resin casts

The morphometric study did not include ischaemia without reperfusion because these experiments have been reported previously [1,20,21]. Morphometric measurements of oxygenated capillary dimensions were unaffected by Y-27632. Resin cast experiments with Y-27632 under normoxic conditions were therefore considered unnecessary. Resin casts confirmed earlier reports [1] that the shape of the capillary lumen changes during ischaemia and reperfusion. Swelling of the endothelial cells during reperfusion resulted in narrowing of capillary lumina and zones of no reflow, which in turn prevented infiltration of many capillaries with resin. Without the endothelial cell swelling which accompanies reperfusion [1], ischaemic capillaries were infiltrated more easily with resin and the rupturing of capillaries observed in reperfused microvasculature did not occur.

Morphometric observations confirmed previous findings [1], that during ischaemia, the significant total capillary diameter decrease is the result of a decrease in luminal diameter; the cross-sectional area of endothelial cells remains the same. This mechanism differs from that reported in skeletal muscle where capillary dimensions are determined by local transmural pressure [31]. Lee et al. demonstrated that average endothelial cell thickness and cell volume decreased with an increase in transcapillary pressure suggesting that a ‘passive recoil’ mechanism induced cell shape change. Folding of the luminal membrane into vesicles and fusion of vesicle and luminal membranes accommodated the fluctuations in exposed luminal membrane area, required by changes in endothelial cell shape. In this way total luminal membrane area was maintained.

In the present study, the cytoplasm of well-oxygenated CCECs contained numerous vesicles, some of which are attached to the luminal membrane. We were unable to find evidence of the type of vesicular membrane folding described by Lee et al. [31] in CCECs. We conclude that this is an inherent difference between skeletal capillary endothelial cells and those in the cardiac microvasculature. This may be a consequence of the differences between the two types of myocytes [32,33] due to energy requirements of sustained mechanical activity of the myocardium. The cardiac microvasculature also differs in pericyte distribution. In muscle, pericytes regulate blood flow by capillary constriction [32,33] and endothelial cells are not reported to regulate blood flow by active ‘self-contraction’. In the cardiac microvasculature, however, pericyte coverage is only 11% while pericytes cover ~30% of capillaries in other tissue types [19]. In addition, the association between pericytes and endothelial cells in cardiac microvasculature is considerably less elaborate than in other tissues such as skeletal muscle [32] where secondary pericyte processes are intimately associated with endothelial cells [33]. Secondary pericyte processes do not occur in the cardiac microvasculature. Consequently, pericyte contraction in the cardiac microvasculature is vestigial [32,33], implying that the role attributed to skeletal muscle pericytes is fulfilled by an alternative mechanism. We suggest that CCECs change shape, via an active mechanism involving ROCK1, to compensate for the reduced presence and contractile potential of pericytes. This is supported by the observation that in hypoxic capillaries, the luminal perimeter and cross-sectional area decrease against an increase in perfusion pressure, induced by the hypoxic conditions [29]. In these conditions the passive recoil mechanism observed by
Lee et al. [31] in skeletal muscle capillaries would induce an increase in luminal perimeter and cross-sectional area. The opposite occurs in cardiac capillaries and we conclude that, in CCECs, the passive recoil mechanism does not play a significant part in cell shape change.

In the present study, without Y-27632, luminal perimeter decreased markedly in the ischaemic/reperfused capillaries in comparison with control capillaries. The precise mechanism causing this reduction in luminal perimeter is unclear. It must, however, involve an active process. If this process was passive, folds would have accounted for the superfluous perimeter, producing a corrugated luminal perimeter [33]. Perimeter length would have remained as that in the control group. In our experiments, luminal perimeter shortened significantly, suggesting that during ischaemia/reperfusion segments of the luminal membrane were internalised and/or released as blebs. These processes are energy driven; for example, blebbing requires myosin light chain phosphorylation and ROCK1 to induce an actomyosin contractile force [17]. In our experiments, evidence of blebbing was absent or negligible with ROCK1 inhibition. Cell dimensional changes induced by ischaemia/reperfusion were prevented by ROCK inhibition. Because ROCK plays a significant role in the contractile mechanism of endothelial cells, our results are more consistent with endothelial cell contraction rather than an external influence such as compression by the myocardium. In addition, hearts exposed to this inhibitor demonstrated enhanced activity on reperfusion. Contractions were noticeably more robust and regular in comparison to reperfused hearts in which ROCK was not inhibited, suggesting that damage in the latter was more severe.

ROCK plays no part in regulation of actomyosin contraction in cardiac myocytes as it does in smooth muscle [10] and inhibition by Y-27632, therefore, does not influence myocyte contraction. Manciet et al. [34] suggested that ‘no reflow’ was caused by compression of the capillaries by the surrounding myocytes in response to ischaemia. Consequently, microvascular compression by myocytes surrounding the capillaries would be expected during ischaemia with this inhibitor applied as in our experiments. Morphometry, however, as well as the morphology of the endothelial cells perfused with Y-27632 and subjected to ischaemia/reperfusion demonstrated that endothelial cell dimensions were essentially the same as oxygenated controls. On reperfusion, each heart beat vigorously and we were unable to find evidence of no reflow in the resin casts.

We have shown that the Rho pathway plays a significant part in changing capillary dimensions via active endothelial cell shape change in the cardiac microvasculature, beyond that required for permeability regulation [25]. Myocyte contraction, damage by free radicals, leukocyte plugging and other factors contribute to reperfusion injury and ‘no reflow’. Active changes in endothelial cell dimensions, however, play a major role in the development of severely damaged microvasculature and myocardium, since inhibiting the contractile mechanism in CCECs can significantly reduce this damage. Targeting the Rho-regulated actomyosin contractile system in the cardiac microvasculature is therefore a useful strategy to prevent or ameliorate ‘no reflow’ and reperfusion injury.

Acknowledgements

This work was carried out in the William Harvey Research Institute. Supported by the British Heart Foundation and Welfide Corporation Ltd.

References


