

# Role of blood cells in ischaemia–reperfusion induced endothelial barrier failure

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Received 8 January 2010; revised 4 March 2010; accepted 12 March 2010; online publish-ahead-of-print 17 March 2010

Ischaemia and reperfusion (I/R) elicits an acute inflammatory response that is characterized by the recruitment of inflammatory cells, oxidative stress, and endothelial barrier failure. Over the past three decades, much progress has been made in our understanding of the mechanisms that underlie the inflammatory response and microvascular dysfunction associated with I/R. This review is focused on the role of leucocytes (neutrophils and T-lymphocytes) and platelets, and their activation products, as mediators of I/R-induced endothelial barrier failure. The contributions of cytokines, chemokines, and oxidative stress to I/R-induced barrier dysfunction are also discussed. It concludes with an analysis of how risk factors for cardiovascular disease, i.e. hypertension, diabetes, hypercholesterolaemia, and obesity, influence the vascular permeability response to I/R. Areas of uncertainty and controversy in this field of investigation are also identified.

**Keywords** Inflammation • Leucocyte-endothelial cell adhesion • Oxidative stress • Platelets • Cardiovascular risk factors

This article is part of the Spotlight Issue on: Microvascular Permeability

## 1. Introduction

Endothelial barrier dysfunction is a well-recognized response of the microvasculature to different pathological conditions that are associated with inflammation. The increased vascular permeability can lead to excess filtration of fluid and proteins, resulting in interstitial oedema and impairment of organ function. The hyperpermeability state generally does not result from overt endothelial cell injury or detachment of endothelial cells from the vessel wall. Instead, the response reflects more subtle changes in the fine structure of the endothelial monolayer, such as a widening of the endothelial paracellular junctions that results from the dissociation of junctional proteins and/or cytoskeletal contraction. The endothelial hyperpermeability that accompanies inflammation has been attributed to a variety of soluble mediators, released from resident and/or circulating inflammatory cells, which engage with specific endothelial cell receptors and consequently open the paracellular pathways for fluid and solute exchange. Other physicochemical factors and processes such as shear rate and the adhesion and transendothelial migration of leucocytes have also been implicated in the endothelial barrier dysfunction associated with inflammation.<sup>1–4</sup>

Ischaemia and reperfusion (I/R) has been implicated in a variety of clinical conditions including thrombolytic therapy for stroke and myocardial infarction, organ transplantation, and the multiple organ dysfunction syndrome.<sup>2</sup> Reperfusion of previously ischaemic tissue, while essential for the prevention of irreversible tissue injury, elicits

a response in the microvasculature which is very similar to inflammation, i.e. it results in an increased production of reactive oxygen species (ROS) and soluble inflammatory mediators, enhanced adhesion of leucocytes and platelets to vascular endothelium and an increased microvascular permeability. These vascular responses, coupled to the injury inflicted on non-vascular cells, result in the phenomenon of reperfusion injury, which can lead to impaired organ function.<sup>5</sup> Although a variety of mediators and mechanisms have been implicated in reperfusion injury, a large and growing body of evidence supports a role for specific blood cell populations in the accompanying microvascular dysfunction. This review is focused on the contribution of leucocytes and platelets to the endothelial barrier failure that is associated with I/R. Some attention is also devoted to the influence of cardiovascular risk factors, which predispose tissues to ischaemic tissue injury, on the endothelial barrier responses to I/R.

## 2. Leucocytes and platelets are recruited into post-ischaemic microvessels

Different approaches have been used to demonstrate that a variety of blood cell populations adhere in the vasculature and emigrate into the perivascular compartment of tissues exposed to I/R (Table 1). Most attention has been devoted to analysing the infiltration of leucocytes,

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**Table 1** Ischaemia–reperfusion elicits the recruitment of different blood cell populations and endothelial barrier dysfunction in different tissues

	Neutrophils	T-lymphocytes	Monocytes	Platelets	Endothelial barrier dysfunction
Heart	76	77	78	79	80,81
Intestine	82	41	83	84	38
Lung	85	86	87	—	88,89
Brain	90	91	92	9	93
Skeletal muscle	94	—	95	—	96
Kidney	97	98	99	7	100
Liver	101	102	103	104	105
Testis	106	—	—	—	—
Retina	107	—	108	109	—

All numbers are relevant references.

particularly neutrophils, into post-ischaemic tissues. In some vascular beds (e.g. gut and lung), neutrophils are observed to adhere in post-capillary venules within minutes after reperfusion, whereas hours to days are required to detect significant neutrophil recruitment in other organs (e.g. brain). Lymphocytes have also been detected in a variety of post-ischaemic organs, including lung, intestine, brain, kidney, and liver. This response generally reflects the accumulation of CD4+ and/or CD8+ T-lymphocytes; however, other mononuclear leucocyte populations have been detected, including B-lymphocytes, monocytes, natural killer cells, and Foxp3+ regulatory T-cells, which are known to blunt immune responses. The accumulation of non-neutrophilic leucocyte populations is typically detected by histopathology after days or weeks of reperfusion, however the trafficking of these blood cell populations has been detected as early as 30 min (gut) to 24 h (brain) after reperfusion. The time dependency of the population-specific recruitment of leucocytes is evident in cerebral microvessels, for which it is estimated that neutrophils account for the majority (~85%) of the total adherent leucocytes detected at 4 h following reperfusion, whereas neutrophils account for only about 20% of total adherent leucocytes (which have more than doubled) at 24 h after reperfusion.<sup>2,6</sup>

The recruitment of leucocytes in post-ischaemic microvessels is often accompanied by the accumulation of platelets (Table 1). Whereas the kinetics of platelet accumulation can differ between regional vascular beds, typically the recruitment of platelets either parallels or precedes that observed for leucocytes. For example, significant platelet accumulation is observed as early as 30–45 min after reperfusion in kidney,<sup>7</sup> liver,<sup>8</sup> and brain.<sup>9</sup> The temporal relationship between leucocyte and platelet accumulation in post-ischaemic tissues has led to the proposal that the two recruitment processes are interdependent. This assertion is supported by evidence that platelet accumulation in post-ischaemic venules is dependent on leucocyte adhesion as well as studies showing that leucocyte adhesion after I/R requires the presence of platelet-associated P-selectin. It is estimated that following I/R approximately 25% of the platelets bind directly to venular endothelium, whereas the remaining 75% of the adherent platelets are attached to leucocytes (primarily neutrophils) that are bound to the vessel wall.<sup>10</sup> Direct visualization of the recruitment processes by intravital microscopy reveals that the two blood populations do bind to one another on the vessel wall and that interference with the binding of one cell population (e.g. leucocytes) to

vessel wall reduces binding of the other (e.g. platelets). The significance of these cell–cell interactions relative to I/R injury remains unclear. However, it may allow for leucocytes to inflict more damage after I/R because of the increased capacity of neutrophils with attached platelets to produce superoxide<sup>11</sup> and platelet-activating factor<sup>12</sup> than either cell is capable of producing alone. In addition to neutrophil–platelet interactions, it has been demonstrated that mice that are genetically deficient in either CD4+ or CD8+ T-lymphocytes exhibit a significantly blunted platelet recruitment response to I/R.<sup>13</sup> It is not clear whether this effect reflects a direct interaction between platelets and T-cells or whether the latter cells exert their effects indirectly by limiting the recruitment of adherent neutrophils in post-ischaemic microvessels.

### 3. Endothelial barrier dysfunction in post-ischaemic tissues

Evidence for impaired endothelial barrier function following I/R has been reported for a variety of tissues (Table 1). A number of different experimental approaches have been used to assess barrier function after I/R, including estimates of the osmotic reflection coefficient for plasma proteins using a lymphatic flux analysis,<sup>14</sup> single-vessel hydraulic conductivity (Lp),<sup>15</sup> electron microscopic evaluation of horseradish peroxidase (HRP) leakage,<sup>16</sup> and the leakage of Evans Blue dye<sup>17</sup> or fluorescently labelled macromolecules.<sup>18</sup> Although the findings generated from these studies are generally interpreted as reflecting changes in vascular permeability, caution should be given to the possibility that, under some circumstances, the demonstration of increased solute extravasation may not reflect a corresponding change in the restrictive properties of the endothelial barrier to solutes but rather increased diffusive and/or convective fluxes of the solute across a normal barrier.

The increased microvascular permeability induced by I/R appears to reflect the cumulative toll of the barrier changes that occur during both the ischaemic and reperfusion phases. For example, exposure of the small bowel to 1 h of ischaemia results in a doubling of vascular permeability to plasma proteins, while the same period of ischaemia following by reperfusion results in a five-fold increase.<sup>5</sup> Similarly, the hydraulic conductivity of mesenteric venules is increased over three-fold above baseline by 45 min of ischaemia, whereas a six- to eight-fold increase is

observed after reperfusion.<sup>19</sup> The results of several studies (summarized below) indicate that the ischaemia and reperfusion components of the barrier dysfunction caused by I/R are distinct and involve different mechanisms and that the reperfusion phase is not merely a delayed manifestation of endothelial 'injury' that is incurred during the ischaemic period.<sup>20</sup> However, the relative contributions of reperfusion to the overall barrier dysfunction induced by I/R is likely to diminish as the duration of the ischaemic period increases. The notion that there are distinct contributions of ischaemia and reperfusion to the barrier dysfunction is also supported by *in vitro* studies demonstrating that endothelial cell monolayers exposed to hypoxia exhibit a 50% increase in albumin permeability, while the same duration of hypoxia followed by reoxygenation yields a 2.3-fold increase.<sup>21</sup>

Direct visualization of protein extravasation after reperfusion of an ischaemic tissue reveals a very rapid permeability response that is evident within minutes after reperfusion. Electron microscopic evaluation of HRP leakage across mesenteric venules after I/R is consistent with impaired endothelial barrier function as early as 30 min after reperfusion, and the HRP leakage appears to preferentially occur across endothelial junctions that exhibit significant leucocyte emigration.<sup>16</sup> Rapid impairment of the endothelial barrier after I/R is also supported by measurements of Lp in single mesenteric venules, which exhibit large increases (above that induced by ischaemia alone) within 60 min of reperfusion.<sup>19</sup> This study also revealed a biphasic water permeability response to I/R, with an initial transient increase (six-fold above baseline) observed 1 h after reperfusion and a second, more sustained increase (eight-fold) in Lp noted at 3 h after reperfusion. Although some adherent leucocytes were noted in the venules during the initial minutes of reperfusion, a larger population of adherent leucocytes was detected during the second phase of the increased Lp after I/R. On the basis of the overlap in kinetics of leucocyte recruitment with the second phase changes in Lp and other more direct evidence, the authors proposed that adherent leucocytes were largely responsible for the second, larger increase in Lp, while a different mechanism underlies the initial increase in Lp after I/R.<sup>19</sup>

#### 4. Mediators of I/R-induced blood cell recruitment and endothelial barrier dysfunction

A number of different cell populations are activated when ischaemic tissues are reperfused with well-oxygenated blood. Cells comprising the wall of blood vessels (e.g. endothelial cells) as well as cells residing in the perivascular compartment (e.g. mast cells, macrophages) show signs of activation following I/R. Endothelial cells assume an inflammatory phenotype following activation, which is characterized by an enhanced production of ROS, release of inflammatory cytokines (e.g. IL-8) and an increased expression of adhesion molecules that bind leucocytes and platelets. The oxidative stress experienced by post-ischaemic endothelial cells appears to underlie the increased production and expression of adhesion molecules via activation of the nuclear transcription factor NFκB.<sup>22</sup> ROS (superoxide and hydrogen peroxide) can also promote inflammation and endothelial barrier dysfunction by (i) eliciting the production of platelet-activating factor, via phospholipase activation; (ii) promoting the activation and deposition of complement on the endothelial cell surface; (iii) mobilizing the stored pool of P-selectin to the endothelial cell surface, where

it mediates leucocyte rolling; (iv) inactivating nitric oxide (NO), and eliciting the activation of perivascular cells, such as mast cells.<sup>1–5</sup>

There are several potential sources of ROS in endothelial cells, including xanthine oxidase, NADPH oxidase, mitochondria, and uncoupled endothelial nitric oxide synthase (eNOS). Of these, xanthine oxidase and NADPH oxidase have received the most attention. Many of the phenotypic changes in endothelial cell function that have been described from *in vivo* experiments have been recapitulated in monolayers of cultured endothelial cells exposed to hypoxia and reoxygenation, including the activation of xanthine oxidase, increased ROS production, NFκB activation, increased adhesion molecule expression, and impaired barrier function.<sup>23</sup> Pharmacological inhibition of xanthine oxidase or treatment with superoxide dismutase blocks the reoxygenation-induced barrier dysfunction *in vitro*.<sup>21</sup> *In vivo* studies of I/R-induced microvascular protein and water permeability also implicate xanthine oxidase-derived ROS in the impaired barrier function.<sup>19,24</sup> However, since xanthine oxidase inhibition or ROS scavenging has also been shown to largely prevent the recruitment of adherent leucocytes, it is not entirely clear from these observations whether the ROS directly alter barrier function or do so indirectly by limiting the adhesion of leucocytes to endothelial cells.<sup>25</sup>

Although less attention has been devoted to NADPH oxidase as a source of the ROS that mediates leucocyte recruitment and impaired barrier function following I/R, there is evidence to support a role for this enzyme in post-ischaemic lung and brain.<sup>25,26</sup> Mouse lungs exposed to I/R exhibit enhanced neutrophil infiltration, lipid peroxidation (a consequence of enhanced ROS production), and increased vascular permeability. However, in wild-type (WT) mice treated with the NADPH oxidase inhibitor apocynin or mice genetically deficient in p47<sup>phox</sup>, a subunit of the NADPH oxidase protein complex, the lung responses to I/R are significantly attenuated.<sup>25</sup> Bone marrow chimeras produced by the transplantation of bone marrow from p47<sup>phox</sup> deficient into WT recipients (or vice versa) reveal that bone marrow-derived cells, rather than endothelial cells, mediate the I/R-induced, NADPH oxidase-dependent responses in the lung vasculature.<sup>16</sup> Brain I/R also results in impaired endothelial barrier function that is dependent on NADPH oxidase, as evidenced by experiments showing reduced blood–brain barrier (BBB) dysfunction after focal ischaemia stroke and reperfusion in NADPH oxidase (gp91<sup>phox</sup>) deficient mice and in WT mice treated with apocynin.<sup>26</sup> Although bone marrow chimeras were not employed to distinguish between the contribution of blood cell and endothelial cell NADPH oxidase, it was shown that monolayers of cultured brain endothelial cells exposed to hydrogen peroxide exhibited a blunted permeability response when treated with apocynin.<sup>26</sup>

An important pathophysiological consequence of increased superoxide production in vascular endothelium subjected to I/R is inactivation of NO. Physiological levels of NO play an important role in preventing cell–cell interactions such as leucocyte–endothelial cell adhesion as well as the binding of platelets with each other and to other cells (endothelial cells, leucocytes).<sup>27</sup> NO donors have been shown to blunt the recruitment of adherent leucocytes, reduce the formation of platelet–leucocyte aggregates, and diminish albumin leakage in post-ischaemic venules.<sup>28</sup> Similar protection against I/R-induced inflammation and endothelial barrier function has been noted in mice that genetically overexpress eNOS.<sup>29</sup> Treatment of non-ischaemic tissues with NOS inhibitors, on the other hand, can reproduce most of the microvascular alterations induced by I/R, including enhanced leucocyte recruitment and increased vascular

permeability.<sup>30</sup> However, the magnitude of the responses observed in post-ischaemic tissues treated with an NOS inhibitor does not differ from those observed with either treatment alone, suggesting that NO production is largely inhibited or most of the NO is inactivated in post-ischaemic microvessels. Although these studies suggest that NO normally protects against endothelial barrier failure, there is also evidence indicating that NO directly diminishes barrier function when leucocytes are not present. The incongruent permeability responses to NO can be explained by measurements of Lp in NOS inhibitor-treated mesenteric venules perfused with or without blood-borne constituents. In the absence of blood-borne constituents, an ~50% reduction in Lp is noted, whereas Lp increases by >75% in vessels perfused by blood during exposure to the NOS inhibitor.<sup>31</sup>

Mast cells residing in the perivascular space also appear to contribute to the leucocyte–endothelial cell adhesion and impaired endothelial barrier function elicited by I/R.<sup>1</sup> Approximately 30% of mast cells surrounding post-capillary venules degranulate following I/R.<sup>28</sup> Mast cell degranulation is generally accompanied by the production and release of superoxide, amines (histamine, serotonin), and cytokines (e.g. TNF- $\alpha$ , IL-1), all of which can promote the recruitment of leucocytes and diminish endothelial barrier function. A role for mast cell degranulation in I/R-induced microvascular dysfunction is supported by reports describing an attenuating influence of mast cell stabilizing drugs or a genetic deficiency of mast cells on leucocyte recruitment and vascular permeability after I/R in the intestine and brain, but not in skeletal muscle.<sup>1,32,33</sup> For example, the impaired BBB function and neutrophil recruitment that occur in brain following middle cerebral artery occlusion and reperfusion is significantly blunted in WT rats treatment with a mast cell stabilizer (cromoglycate) and in mast cell deficient rats.<sup>33</sup>

Macrophages that reside in the perivascular compartment have also been implicated in the leucocyte recruitment and impaired barrier function induced by I/R. Activated macrophages produce and release a variety of cytokines, chemokines as well as reactive oxygen and nitrogen species that can increase endothelial adhesivity to blood cells and reduce endothelial barrier function. Macrophage depletion studies in lung and intestine indicate less accumulation of inflammatory mediators (cytokines, chemokines) and neutrophils after I/R compared with untreated animals.<sup>34–36</sup> These anti-inflammatory actions of macrophage depletion are also associated with a significant reduction in I/R-induced vascular permeability.<sup>34,36</sup>

## 5. Evidence implicating blood cells in I/R-induced endothelial barrier failure (Figure 1)

### 5.1 Neutrophils

There are several lines of evidence suggesting that neutrophil accumulation in post-ischaemic microvessels is a cause rather than a consequence of I/R-induced endothelial barrier failure. In some tissues (e.g. mesentery, brain), a strong positive correlation exists between the magnitude of the I/R-induced increase in vascular permeability and the number of adherent and/or emigrated neutrophils in/around post-capillary venules.<sup>28,37</sup> Direct evidence for the involvement of neutrophils is provided by studies demonstrating that animals rendered neutropenic exhibit a blunted vascular permeability response to I/R.<sup>38–40</sup> Similarly, it has been demonstrated that immune blockade

of adhesion molecules (P-selectin, ICAM-1, CD11/CD18) that mediate the rolling, firm adherence and/or emigration of neutrophils also diminish the endothelial barrier failure associated with I/R.<sup>38,40</sup>

### 5.2 T-lymphocytes

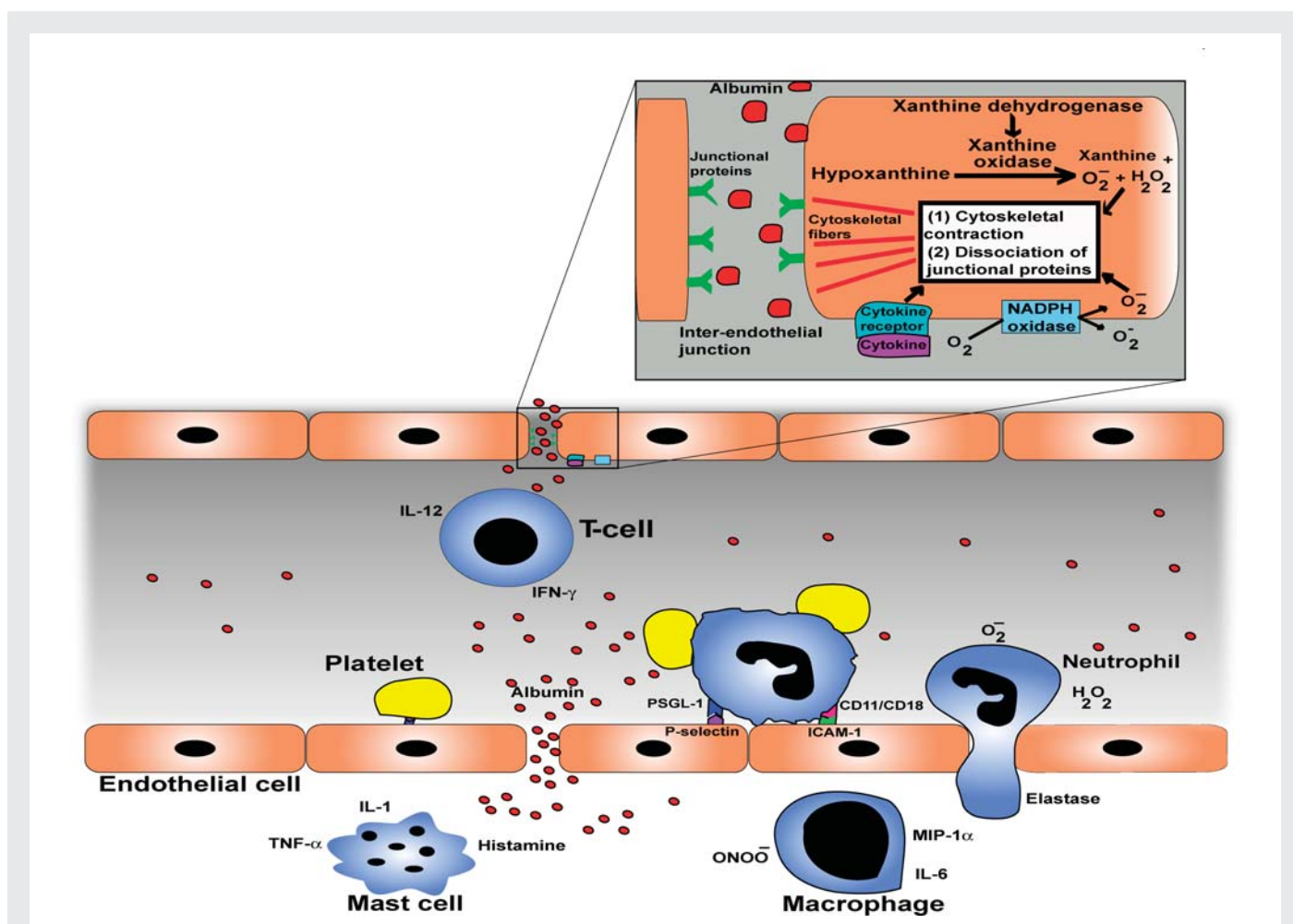
CD4+ and CD8+ T-lymphocytes are recruited into post-capillary venules of the small intestine in WT mice following I/R and this recruitment of T-cells is associated with an increased vascular permeability to albumin.<sup>41</sup> The pathophysiological relevance of the T-cell recruitment is evidenced by the observation that immunodeficient SCID mice (which lack T-cells) exhibit a significantly blunted vascular permeability response to I/R, but the permeability response can be restored to WT levels when SCID mice are reconstituted with T-cells from WT mice.<sup>41</sup> A similar role for T-lymphocytes as a mediator of I/R-induced endothelial barrier failure has been reported in the renal vasculature, where WT mice, but not mice deficient in CD3+ T-cells, exhibit an increased vascular permeability, and adoptive transfer of WT T-cells into the mutant mice restores the permeability response.<sup>42</sup> Immunoblockade of CD4+, but not CD8+, T-cells has been shown to reduce the lung vascular permeability response to I/R, although the barrier compromising effect of the CD4+ T-cells may have resulted from their modulating influence on neutrophil recruitment.<sup>17</sup>

### 5.3 Platelets

Less attention has been devoted to evaluating the contribution of platelet accumulation to I/R-induced endothelial barrier failure. The few reports that address this issue suggest that the presence of platelets either do not alter or improve endothelial barrier function. For example, one study of the post-ischaemic coronary vasculature indicates that platelets are not requisite for the barrier dysfunction, whereas another report describes an improved barrier function following the addition of platelets.<sup>43,44</sup> A protective effect of platelets has also been reported for the lung.<sup>45</sup> A limitation of all these studies is that the organs used to assess the permeability responses to I/R were perfused with artificial solutions, to which platelets are added. *In situ*, blood perfused tissues may yield a different contribution of platelets to the I/R-induced barrier dysfunction.

## 6. Blood cell-derived mediators of I/R-induced endothelial barrier failure

Leucocytes and platelets produce and release a variety of compounds that can diminish the barrier function of endothelial cells (Table 2). The contribution of these activation products to the blood cell-dependent endothelial barrier dysfunction elicited by I/R remains uncertain. However, there are a few studies that directly implicate blood cell-derived products in the vascular permeability responses to I/R. As mentioned above, bone marrow chimeras produced from NADPH oxidase knockout mice provide support for blood cell-derived superoxide as a mediator of the pulmonary endothelial barrier failure associated with I/R.<sup>25</sup> Since both leucocytes and platelets can produce superoxide via NADPH oxidase, the specific cellular source cannot be identified from the chimera results; however, leucocytes are the more likely candidate because of their capacity to generate much more superoxide per cell.<sup>46</sup>



**Figure 1** Mechanisms of endothelial barrier failure following ischaemia–reperfusion (I/R). The recruitment of leucocytes and platelets into post-capillary venules is enhanced by I/R. The products of leucocyte, platelet, and endothelial cell activation ultimately lead to a widening of the inter-endothelial junctions secondary to dissociation of junctional proteins and/or cytoskeletal contraction. Potential mediators of the inflammatory response and barrier failure include interleukin-12 (IL-12), interferon- $\gamma$  (IFN- $\gamma$ ), superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1), histamine, interleukin-6 (IL-6), and macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ).

The roles of cytokines and chemokines to I/R-induced endothelial barrier failure have also been addressed, although few studies have directly assessed the contribution of blood cells to this response. TNF- $\alpha$  has been implicated in the I/R-induced endothelial barrier dysfunction observed in lung, skeletal muscle, and brain.<sup>47–49</sup> A contribution of TNF- $\alpha$  to I/R-induced BBB dysfunction has been demonstrated using monolayers of cultured brain endothelial cells exposed to simulated flow cessation and reperfusion.<sup>50</sup> The TNF- $\alpha$ -dependent permeability response was noted both in the presence and absence of leucocytes. However, more of the cytokine was generated in the presence of leucocytes and TNF- $\alpha$  immunoblockade exerted a more profound protection against the shear stress-dependent endothelial barrier dysfunction, suggesting a major role for leucocyte-derived TNF- $\alpha$ .

Both alpha (MIP-2, CINC) and beta (MIP-1, MCP-1, RANTES) chemokines have been implicated in the increased vascular permeability elicited by I/R. In the pulmonary microvasculature, immunoblockade of MIP-1, MIP-2, and CINC blunts the I/R-induced permeability response, whereas RANTES and MCP-1 antibodies do not afford protection.<sup>51,52</sup> Antisense oligonucleotides as well

as neutralizing antibodies directed against the chemokine CCL2 have been shown to blunt the BBB permeability response to focal cerebral I/R.<sup>53</sup> This observation, coupled to studies demonstrating endothelial cells deficient in CCR2 (the receptor for CCL2) are resistant to I/R-induced BBB failure,<sup>54</sup> suggest a major role for CCL2–CCR2 complex activation in the regulation of BBB function.

The role of blood cell-derived RANTES as a mediator of I/R-induced BBB dysfunction has been recently addressed.<sup>55</sup> This chemokine, which promotes the directed migration of leucocytes into damaged or inflamed tissue, is produced by a variety of cells, including T-lymphocytes, platelets, endothelial cells, smooth muscle cells, and glial cells. The exaggerated leucocyte and platelet adhesion, and increased BBB permeability elicited in WT mice after focal cerebral I/R are significantly blunted in RANTES<sup>-/-</sup> mice. Similar attenuation of the I/R-induced responses are noted in chimeras produced by transplantation of bone marrow from RANTES<sup>-/-</sup> mice into WT recipients, suggesting that blood cells are the likely source of the chemokine. Since immunodeficient Rag-1<sup>-/-</sup> mice exhibit the same robust increase in plasma RANTES concentration as WT

mice, T-lymphocytes are an unlikely source of the chemokine released following I/R. Platelets, which release RANTES upon activation, may be the major blood cell source of the chemokine.<sup>55</sup>

CD40/CD40 ligand (CD40L) signalling has been implicated in a variety of acute and chronic inflammatory conditions. Mice that are

**Table 2 Activation products released by leucocytes and platelets that may impair endothelial barrier function**

Leucocytes	Platelets
Reactive oxygen species <sup>110</sup>	Reactive oxygen species <sup>111</sup>
Superoxide	Superoxide
Hydrogen peroxide	Hydrogen peroxide
Cytokines/chemokines <sup>112</sup>	Cytokines/chemokines <sup>112–114</sup>
Interleukins-1, -2, -6, -8, -12	Interleukins-1, -7, -8
Interferon-alpha, -gamma	RANTES (regulated upon activation, normal T-cell expressed and secreted)
Tumour necrosis factor-alpha, -beta	Tumour necrosis factor-beta
Transforming growth factor-beta	CD40 ligand
Monocyte chemoattractant factor-1	
Proteases <sup>115</sup>	Growth factors <sup>113</sup>
Cathepsin-G	Platelet-derived growth factor
Elastase	Transforming growth factor-beta
Collagenase	Vascular endothelial growth factor
Oxidases <sup>115</sup>	Lipid mediators <sup>114,116</sup>
Myeloperoxidase	Thromboxane A2
	12-HETE
Lipid mediators <sup>113</sup>	Procoagulants <sup>113,117</sup>
Leukotrienes B4, C4	Thrombin
Platelet activating factor	Adenosine di- and tri-phosphates
	Platelet factor-4
Miscellaneous <sup>115</sup>	
Cationic proteins	
Histamine	
Vascular endothelial growth factor	

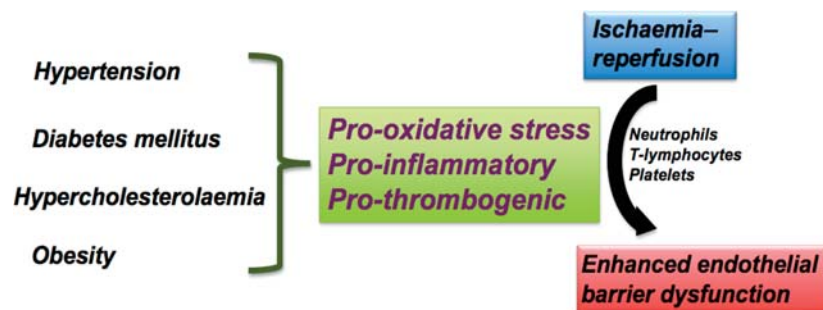
genetically deficient in either CD40 or CD40L exhibit significantly blunted blood cell (leucocyte and platelet) adhesion and vascular permeability responses to focal cerebral I/R.<sup>56</sup> Although the cellular sources of CD40 and CD40L that mediate the altered BBB function were not directly addressed, it was proposed that the engagement of CD40 would promote the recruitment of activated adherent leucocytes, which release oxygen radicals, matrix metalloproteinases, and/or other factors that increase BBB permeability. CD40/CD40L signalling has also been implicated in I/R-induced increases in pulmonary microvessel Lp, via a mechanism that involves T-lymphocytes.<sup>57</sup> Since activated platelets shed large amounts of soluble CD40L (sCD40L) into plasma, and sCD40L retains biological function, it is possible that sCD40L contributes to both the local and distant organ vascular permeability responses elicited by I/R.<sup>2</sup>

## 7. Modulating influence of risk factors for cardiovascular disease

Much of what is known about the pathobiology of I/R-induced microvascular dysfunction, inflammation, and tissue injury has been derived from studies on animals that are otherwise normal. However, epidemiological evidence in humans clearly demonstrates that the incidence of ischaemic tissue disease is largely determined by the presence of certain risk factors, including hypertension (HTN), hypercholesterolaemia (HCh), diabetes mellitus, obesity, and cigarette smoke, with combinations of risk factors (e.g. HTN & HCh) exerting a synergistic effect on the incidence of ischaemic disease. However, relatively little attention has been devoted to determining whether these risk factors (either alone or in combination) merely increase the likelihood that a tissue experiences an ischaemic episode (e.g. atherosclerosis-induced flow restriction) or whether the risk factors also render the microvasculature more sensitive to the deleterious effects of I/R, resulting in the recruitment of more inflammatory cells and platelets, and larger increases in vascular permeability following a given ischaemic stress. The latter possibility appears tenable in view of evidence that the risk factors induce a pro-oxidative, pro-inflammatory, and pro-thrombotic environment (Figure 2).

### 7.1 Hypertension

Studies of the mesenteric microcirculation in normotensive (WKY) and hypertensive (SHR) rats after I/R have revealed no differences



**Figure 2** Role of risk factors for cardiovascular disease in ischaemia/reperfusion (I/R)-induced endothelial barrier failure. Hypertension, diabetes mellitus, hypercholesterolaemia, and obesity induce a pro-oxidative, pro-inflammatory, and pro-thrombotic environment, mediated at least in part by blood cells (e.g. neutrophils, T-lymphocytes, and platelets), that exacerbates the I/R-induced endothelial barrier dysfunction.

in the I/R-induced recruitment of leucocytes and platelets, whereas a more pronounced albumin extravasation occurs across venules in SHR.<sup>58</sup> Retinal microvessels of SHR exhibit a larger leucocyte recruitment response in venules and more evidence of tissue damage after I/R than their normotensive counterparts.<sup>59</sup> Transient ischaemia has been shown to increase BBB permeability in stroke-prone spontaneously hypertensive rats,<sup>60,61</sup> however, it remains unclear whether this response is associated with (or linked to) an increased recruitment of blood cells into the cerebral microcirculation.

## 7.2 Hypercholesterolaemia

An exaggerated leucocyte recruitment response to I/R has been demonstrated in both genetic and diet-induced models of HCh. This phenomenon has been demonstrated in post-ischaemic mesenteric,<sup>62</sup> skeletal muscle,<sup>63</sup> and cerebral venules.<sup>64</sup> Mesenteric and skeletal muscle microvessels also exhibit a more pronounced I/R-induced increase in vascular permeability with HCh. The risk factor-enhanced barrier failure appears to be directly linked to the leucocyte recruitment, since adhesion molecule-directed antibodies that significantly attenuate leucocyte recruitment also blunt the permeability response. A similar protective effect against the permeability response was noted when interventions were used (e.g. GPIIb/IIIa antagonist) to prevent the formation of homotypic or heterotypic platelet aggregates.<sup>62</sup> Oxidized low-density lipoprotein (oxLDL), which has been implicated in the inflammatory responses associated with HCh, has also been shown to enhance I/R-induced leucocyte adhesion and emigration in mesenteric venules.<sup>65</sup> However, this was not associated with a correspondingly larger vascular permeability response.

## 7.3 Diabetes

Increased vascular permeability and inflammation are characteristic features of the microvascular responses to diabetes mellitus. A variety of factors have been implicated in the endothelial barrier dysfunction associated with diabetes, including hyperglycaemia, vascular endothelial growth factor, and oxidative stress.<sup>66</sup> Diabetic animals exposed to I/R exhibit an exaggerated vascular permeability response that is accompanied by accelerated ROS production and more adherent leucocytes in post-capillary venules.<sup>67,68</sup> Whereas leucocytes have been implicated as a source of ROS in the post-ischaemic microvasculature of diabetic animals,<sup>69</sup> hyperglycaemia-induced overproduction of ROS by mitochondrial electron transport chain may also contribute to the exaggerated permeability response to I/R.<sup>70</sup> PAF and LTB<sub>4</sub> have been implicated in the exaggerated leucocyte recruitment,<sup>67,71</sup> which involves CD11/CD18-ICAM-1 adhesive interactions (firm adhesion) and P-selectin (rolling).<sup>72</sup> Immunoblockade of these adhesion molecules blunts the I/R-induced, diabetes-enhanced endothelial barrier dysfunction, and oxidative stress,<sup>67,72</sup> suggesting a critical role for the adherent leucocytes. NO donating compounds are equally effective in blunting the diabetes-enhanced I/R-induced microvascular dysfunction.<sup>73</sup>

## 7.4 Obesity

The expanded pool of activated adipocytes that accompanies obesity appears to elicit a systemic inflammatory response that is characterized by endothelial cell dysfunction, oxidative stress, and the activation of circulating immune cells. It has been reported that the brain inflammation and BBB dysfunction elicited by sepsis are exacerbated in obese mice compared with their lean counterparts.<sup>74</sup> A similar effect of obesity has been noted following focal cerebral I/R,<sup>75</sup>

where leptin-deficient obese mice exhibit larger increases in leucocyte and platelet adhesion, BBB permeability, water content, and infarct volume compared with lean mice. The post-ischaemic obese mice also exhibited higher plasma levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) than lean mice. Immunoneutralization of MCP-1, but not IL-6, was shown to reduce infarct volume in the obese mice.

## 8. Conclusions

The endothelial barrier failure elicited by I/R is accompanied by oxidative stress and the recruitment of leucocytes and platelets. The blood cells that are recruited into the post-ischaemic microvasculature appear to contribute to both the endothelial barrier dysfunction and enhanced production of ROS, via mechanisms that require adhesive interactions between the blood cells and vascular endothelium. The available evidence suggests that products of blood cell activation, including ROS, cytokines, and chemokines play a major role in mediating the adhesion-dependent increase in vascular permeability caused by I/R. Whether these agents act directly on endothelial cells or do so indirectly by activating perivascular cells such as mast cells and macrophages remain unclear. Despite these uncertainties about the nature of the involvement of different cell populations and molecular mediators in the I/R-induced endothelial barrier dysfunction, there is mounting evidence that the well-established risk factors for cardiovascular disease amplify the inflammatory and oxidative responses elicited by I/R, with a corresponding exacerbation of the barrier failure. Drugs that target blood cell-derived factors may provide a novel therapeutic strategy for restoring endothelial barrier function in clinical conditions associated with permeability oedema.

**Conflict of interest:** none declared.

## Funding

The authors are supported by a grant from the National Heart Lung and Blood Institute (HL26441) of the National Institutes of Health.

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