Inhibition of contractile activation reduces reoxygenation-induced endothelial gap formation

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Abstract

Objective: Barrier function of coronary endothelium becomes disturbed by ischemia–reperfusion. We investigated the mechanism of reperfusion-induced endothelial gap formation in monolayers of cultured endothelial cells (CEC) of the rat, exposed to simulated ischemia (40 min anoxia, pH 6.4) and reperfusion (30 min reoxygenation, pH 7.4). Methods: Cytosolic Ca$^{2+}$ (fura-2) and intercellular gap formation (planimetric analysis) were determined. Reoxygenation conditions were varied: (a) continuing perfusion at pH 6.4, (b) with or without glucose (2.5 mM), (c) in presence of NaCN (2 mM), (d) with Ca$^{2+}$ (10 mM) or BAPTA/AM (25 μM), (e) in the presence of ML-7 (5 μM) or wortmannin (1 μM).

Results: During anoxia, CEC developed cytosolic Ca$^{2+}$ overload which was not reversed during 30 min reoxygenation. Intercellular gap formation started during anoxia, but was increased during reoxygenation. Reoxygenation-related gap formation was largest in presence of glucose, lower when glucose was withdrawn or NaCN was added. Presence of ML-7 or wortmannin also reduced gap formation during reoxygenation. Conclusions: Reoxygenation induces gap formation. This is dependent on (i) Ca$^{2+}$ overload during reoxygenation, (ii) energy production and (iii) activation of myosin light chain kinase. Together these results indicate that activation of the endothelial contractile machinery is the underlying cause.

Keywords: Calcium (cellular); Contractile apparatus; Endothelial function; Ischemia; Reperfusion

1. Introduction

In the ischemic–reperfused heart endothelial permeability is increased, causing edematous swelling of the reperfused tissue [1,2]. Endothelial barrier function is impaired due to the opening of intercellular gaps [3]. A rapid postischemic swelling of myocardium is also observed in saline-perfused hearts [4,5]. This indicates that endothelial permeability in reperfused hearts is due, at least in part, to a mechanism independent of leukocytes or other blood elements. Causes which contribute to the increase in endothelial permeability specifically under reperfusion conditions may be extrinsic to the endothelial cells, such as ischemic metabolites from cardiomyocytes, or intrinsic, i.e., caused by the metabolic disturbance of the endothelial cells themselves. In the present study we focused on causes intrinsic to endothelial cells.

As an experimental model we used cultured monolayers of microvascular coronary endothelial cells to study basic elements of the endothelial permeability response to conditions of simulated ischemia and reperfusion. Previous studies showed that loss of barrier function of endothelial cells can be due to activation of the endothelial contractile machinery [1]. Contractile elements of endothelial cells are, like those of smooth muscle cells, controlled by the phosphorylation state of myosin light chains (MLC) [6,7]. Loss of endothelial barrier function may, therefore, be due to activation of MLC kinase, which represents an energy and Ca$^{2+}$-dependent process [8–10] that leads to contractile activation in endothelial cells and subsequent opening of intercellular gaps. In reoxygenated endothelial cells two
factors come together which favor an activation of the contractile machinery: Ca\(^{2+}\) overload of the cytosol and a resupply of reoxygenation with energy [11]. This lead us to investigate if reoxygenation-induced formation of intercellular gaps is (i) related to cellular Ca\(^{2+}\) overload, (ii) dependent on energy supply and (iii) sensitive to the application of ML-7 or wortmannin, two chemically distinct inhibitors of MLC kinase [12]. We were also interested to see if we could interfere with specific causes at the time of reoxygenation and thereby inhibit reoxygenation-induced gap formation, since this would identify these changes as a form of reperfusion injury.

2. Methods

2.1. Cell cultures

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23 1985). Coronary endothelial cells were isolated from 200- to 250-g male Wistar rats and grown in culture as previously described [13,14]. Briefly, hearts were perfused with collagenase, chopped, and dispersed into a suspension. From this suspension, the fraction of endothelial cells was purified. Cells were plated at a density of 10\(^{5}\) cells on 100-mm plastic petri dishes. Cells were cultured at 37 °C in medium 199 with Earle’s salt, supplemented with 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 20% (v/v) FCS. Medium was changed every second day. Confluent cultures of primary endothelial cells were trypsinized in PBS (composed of (in mM) 137 NaCl, 2.7 KCl, 1.5 KH\(_{2}\)PO\(_4\), at pH 7.4, supplemented with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA), and seeded at a density of 7×10\(^4\) cells/cm\(^2\) on glass coverslips. Experiments were performed with confluent monolayers, 3 days after seeding.

2.2. Experimental protocol

Endothelial cells on glass cover slips were introduced into a perfusion chamber (1 ml filling volume) and were superfused at a flow rate of 0.5 ml/min with a modified Hepes buffer (containing in mmol/l: 140.0 NaCl, 2.6 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1.3 CaCl\(_2\), 2.5 glucose and 25.0 Hepes). pH was 7.4 or 6.4 at 30 °C. Normoxic medium was equilibrated with air. The anoxic medium was glucose-free and equilibrated before and during experiments with 100% N\(_2\), PO\(_2\) in anoxic medium was less than 22 μPa as determined with resazurin [15]. Anoxic and normoxic media were serum-free. The medium was transferred into the perfusion chamber through gas-tight steel capillaries. Under standard conditions cells were exposed to anoxia at pH\(_a\) 6.4 for 40 min followed by 30 min superfusion with normoxic medium with glucose at pH\(_o\) 7.4. Reoxygenation conditions were varied: (a) continuing perfusion at pH\(_o\) 6.4, (b) with or without glucose (2.5 mM), (c) with NaCN (2 mM), (d) with Ca\(^{2+}\) (10 mM) or BAPTA/AM (25 μM), (e) with presence of myosin light chain kinase inhibitors ML-7 (5 μM) or wortmannin (1 μM).

2.3. Ca\(^{2+}\) measurement and determination of intercellular gaps

Cytosolic concentration of Ca\(^{2+}\) was measured with the fluorescent indicator fura-2. For loading with the indicator cells were incubated for 60 min in medium 199 containing 4% FCS and 2.5 μmol/l of the acetoxymethyl ester of fura-2. This was followed by 30 min incubation without fura-2. Fura-2 fluorescence was analyzed using a TILL Photonics imaging system (Martinried, Germany). Excitation was alternated between 340 and 380 nm. Emitted light was detected at 510 nm, and the background was corrected. Fura-2 fluorescence was calibrated according to the methods described by Grynkiewicz et al. [16]. For this purpose, cells were exposed to 5 μM ionomycin in modified Hepes buffer containing either 3 mM Ca\(^{2+}\) or 5 mM EGTA to obtain the maximum (R\(_{max}\)) and the minimum (R\(_{min}\)) of the ratio of fluorescence (R), respectively. [Ca\(^{2+}\)]\(_i\) was calculated according to the equation

\[
[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)
\]

with use of the dissociation constant (K\(_d\)) of fura 2 determined in intact cells [10]; β is the ratio of the 380-nm excitation signals of ionomycin-treated cells at 5 mM EGTA and at 3 mM Ca\(^{2+}\). Simultaneously to the measurement of the fura-2 ratio, intercellular gap formation was quantified by planimetric analysis of the fluorescence images (each 327 680 pixels) obtained at the Ca\(^{2+}\)-independent 360 nm excitation wavelength. Images were binarized by an automatic recognition program which sets the fluorescent areas of cells to 1 and cell-free non-fluorescent areas to 0. For each image the number of pixels with assignment of 0 was determined. Typically, normoxic control images contained 6000–8000 0-pixels, images taken after 40 min anoxia 45 000–55 000 0-pixels. In each experiment the normoxic control value of 0-pixels was counted as 0%, the endanoxic value as 100% gap formation. Changes in reoxygenation were expressed relative to this scale. Experiments (<5% of cases) in which individual cells were lost from the visual field during incubations, were not included in the analysis.

2.4. Materials

Falcon plastic tissue culture dishes were from Becton- Dickinson (Heidelberg, Germany); fura-2-AM was from Molecular Probes (Eugene, OR); ionomycin, ML-7 and wortmannin from Calbiochem (Bad Soden, Germany);
NaCN and glucose were from Merck (Darmstadt, Germany); FCS, medium 199, penicillin–streptomycin, and trypsin–EDTA were from Gibco–Life technologies (Eggengstein, Germany). All other chemicals were of the best available quality, usually analytic grade.

2.5. Statistical analysis

Values are expressed as mean±S.E.M. of n experiments taken from at least four experiments using independent monolayer preparations. Statistical analysis was performed by one-way ANOVA in conjunction with the Student–Newman–Keuls test for post hoc analysis. Between-group analysis was performed, and P values <0.05 were considered significant.

3. Results

Endothelial monolayers were exposed to 40 min anoxia in acidic medium (pH 6.4), to simulate in part ischemic conditions, and were then reoxygenated at physiological pH 7.4, to simulate reperfusion conditions. Fig. 1 (upper panel) shows the time course of intercellular gap formation during anoxia and 30 min reoxygenation. During the first 10 min of anoxia intercellular gaps opened in the monolayer. Opening of gaps was further increased during the 30-min subsequent reoxygenation phase.

Anoxic endothelial cells developed cytosolic Ca$^{2+}$ overload during anoxia, as determined by the increase in ratio fluorescence of the Ca$^{2+}$ indicator fura-2 (normoxic ratio: 1.21±0.05; corresponds to [Ca$^{2+}$]$_i$ of 79±3 nM; end-anoxic ratio: 1.51±0.03, corresponds to [Ca$^{2+}$]$_i$ of 405±8 nM) (Fig. 1, lower panel). Rise of cytosolic Ca$^{2+}$ in anoxic endothelial cells can be divided into a steep initial part and a slower progression later on. We showed in previous studies [11,17] that the first rapid phase of Ca$^{2+}$ rise in anoxic endothelial cells is due to Ca$^{2+}$ release from the endoplasmatic reticulum, and the second to Ca$^{2+}$ entry across the plasmalemma. In reoxygenated endothelial cells, the elevation of cytosolic Ca$^{2+}$ was not reversed within the 30-min observation time. Instead, it increased even further: Fura-2 ratio after 30 min reoxygenation: 1.59±0.04, corresponding to a cytosolic [Ca$^{2+}$]$_i$ of 526±13 nM).

In the applied standard protocol of simulated ischemia–reperfusion conditions, anoxia at pH$_o$ 6.4 is followed by reoxygenation at pH$_o$ 7.4. To analyze the effect of this pH change at the onset of reperfusion, we also performed experiments with a continuation of low pH during reoxygenation (Fig. 2). Continued acidosis had no influence on reoxygenation-induced gap formation but augmented the Ca$^{2+}$ overload during reoxygenation. Control experiments, in which anoxic conditions with low pH$_o$ were extended to the time otherwise occupied by reoxygenation conditions, showed, that reoxygenation has an influence independent of changes in pH$_o$ on cytosolic Ca$^{2+}$ rise and gap formation.

![Graph](image1.png)

Fig. 1. Synopsis of gap formation and Ca$^{2+}$ accumulation during 40 min anoxia and 30 min reoxygenation in a representative experiment. Upper panel, gap area as percentage (%); lower panel, fura-2 ratio in arbitrary units (a.u.).

![Graph](image2.png)

Fig. 2. Effect of post anoxic conditions on gap formation (top) and fura-2 ratio (below). Gap formation is expressed as percentage of end-anoxic value (100%). Fura-2 ratio, indicating changes in cytosolic Ca$^{2+}$, is expressed in arbitrary units (a.u.). Data obtained at the end of the following protocols: A64R74 (dark bar), 40 min anoxia at pH$_o$ 6.4 followed by 30 min reoxygenation at pH$_o$ 7.4; A64R64 (clear bar), 40 min anoxia at pH$_o$ 6.4 followed by 30 min reoxygenation at pH$_o$ 6.4. A64 ctd. (clear bar), 70 min anoxia at pH$_o$ 6.4. Data are mean±S.E.M., n=6–8 experiments, *P<0.05 versus A64 ctd.
During reoxygenation, changes in energy supply conditions influenced the progression in cytosolic Ca\(^{2+}\) overload (Fig. 3). When reoxygenation was performed in presence of glucose (control conditions), the additional rise in cytosolic Ca\(^{2+}\) was only moderate. Ca\(^{2+}\) rise during reoxygenation was enhanced in absence of glucose or when the inhibitor of oxidative phosphorylation NaCN was applied. The largest increase in postanoxic Ca\(^{2+}\) overload was observed when glucose was absent and NaCN present. In this case a doubling of the end-anoxic Ca\(^{2+}\) level was observed within 30 min reoxygenation.

Under the same variation of reoxygenation conditions the enlargement of gaps was monitored (Fig. 4). The changes in gap size were not related to the variations in Ca\(^{2+}\) overload. Reoxygenation-induced gap formation was largest in presence of glucose and with an active oxidative phosphorylation system (control conditions), lower when glucose was withdrawn or NaCN was added. The reoxygenation effect on gaps was entirely blunted when both energy lowering means were applied.

Under metabolic control conditions (glucose present), cytosolic Ca\(^{2+}\) during reperfusion was manipulated during the reoxygenation period. For this purpose the cells were either loaded with the Ca\(^{2+}\) chelator BAPTA or exposed to 10 mM Ca\(^{2+}\) in the incubation media. At 30 min reoxygenation the resulting cytosolic Ca\(^{2+}\) load, indicated by the fura-2 signal, was related to gap formation in a saturable relationship (Fig. 5). Marked reduction of Ca\(^{2+}\) load reduced reoxygenation-induced gap formation, but gaps

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Fig. 3. Effect of metabolic inhibition on fura-2 ratio (a.u.), indicating cytosolic Ca\(^{2+}\) changes, during reoxygenation. Reoxygenation was performed under different experimental conditions: control (with glucose), without glucose; in presence of NaCN with glucose, without glucose. Dark bars represent fura-2 ratio under normoxic conditions and after 40 min anoxia, clear bars represent fura-2 ratio after 30 min reoxygenation. Data are mean ± S.E.M. n = 6–8 experiments, *P < 0.05 versus end-anoxic value.

Fig. 4. Relationship between gap formation and fura-2 ratio after 30 min reoxygenation. Gap formation is expressed as percentage of end-anoxic value (100%). Fura-2 ratio (a.u.) indicates cytosolic Ca\(^{2+}\) changes. Ca\(^{2+}\) load was varied by either loading cells with BAPTA (open circle) or increasing extracellular Ca\(^{2+}\) from 1.3 mM (open square) to 10 mM (open triangle). Data are presented as mean values ± S.E.M. n = 6–8 experiments, *P < 0.05 versus end-anoxic value.

Fig. 5. Effect of metabolic inhibition on gap formation. Reoxygenation was performed under different experimental conditions: control (with glucose), without glucose; in presence of NaCN with glucose, without glucose. Gap formation is expressed as percentage of end-anoxic value (100%). Dark bars represent gap formation under normoxic conditions and after 40 min anoxia, clear bars represent gap formation after 30 min reoxygenation. Data are mean ± S.E.M. n = 6–8 experiments, *P < 0.05 versus end-anoxic value.
Ca\(^{2+}\) overload during the reperfusion period, (ii) energy production and (iii) MLC kinase activation. Together these findings indicate that reoxygenation-induced gap formation is due to an activation of the endothelial contractile machinery.

We showed before that conditions of simulated ischemia cause a rise in cytosolic Ca\(^{2+}\) and an increase in barrier permeability in endothelial monolayers [1,11]. Cytosolic Ca\(^{2+}\) rises in two phases: a first steep rise is due to Ca\(^{2+}\) release from the endoplasmic reticulum and a second due to an influx of exogenous Ca\(^{2+}\) triggered by the initial endogenous Ca\(^{2+}\) release [17]. Acidosis accelerates the accumulation of Ca\(^{2+}\) during the second phase [11]. The function of endothelial monolayers is progressively impaired during ischemic conditions [1,18,19]. This is due to the opening of intercellular gaps, i.e., of paracellular passageways for water, solutes and macromolecules. Under ischemic conditions hyperpermeability of endothelial monolayers develops in two phases: a first rapid rise in permeability is due to activation of the endothelial contractile machinery and a subsequent slow progression of hyperpermeability is due to disintegration of cell adhesion complexes or distributional changes of cytoskeletal proteins [1,20,21].

In the present study the aforementioned changes in cytosolic Ca\(^{2+}\) control and monolayer barrier function were also observed during the initial period of simulated ischemia. It was also consistent with previous work that the cytosolic Ca\(^{2+}\) overload resulting from ischemic conditions is not readily reversible upon simulated reperfusion conditions, i.e., within 30 min reoxygenation and re-normalization of extracellular pH. In fact, reoxygenation was found to aggravate Ca\(^{2+}\) overload, as evident when reoxygenated cells were compared with cells under continued anoxia. The renormalization of extracellular pH during reoxygenation was not the cause for this effect, since reoxygenation at lower pH enhanced Ca\(^{2+}\) overload even further.

Reoxygenation conditions not only increased Ca\(^{2+}\) overload but also gap formation. As this aggravation could be modified by a variation of the reoxygenation conditions, it must be elicited by a factor or a combination of causes induced by the simulated reperfusion conditions. In other words, aggravation of gap formation represents reperfusion injury. Since reoxygenation at pH 6.4 produced the same increment in gap formation as reoxygenation at pH 7.4, the change in extracellular pH associated with reoxygenation conditions is not one of the triggering factors for this form of reperfusion injury (not a ‘pH paradox’).

The changes in cell shape developing during reoxygenation require cytosolic Ca\(^{2+}\) overload, but this dependence is saturable. Under standard conditions with 1.3 mM Ca\(^{2+}\) in normoxic media, the Ca\(^{2+}\) overload after 30 min reperfusion was already in the saturation range. A further increment in cytosolic Ca\(^{2+}\) overload during reoxygenation was without effect on reoxygenation-induced gap forma-
tion. Buffering of intracellular Ca\textsuperscript{2+} during reoxygenation prevented reoxygenation-induced gap formation.

Variation of energy supply conditions during reoxygenation had a marked influence on reoxygenation-induced gap formation. Lack of glucose and inhibition of oxidative phosphorylation by NaCN reduced it. This shows that reoxygenation-induced gap formation is an energy-dependent phenomenon. In these experiments Ca\textsuperscript{2+} changes were opposite to the changes in gap formation, i.e., Ca\textsuperscript{2+} was further increased when energy production during reoxygenation was inhibited. This represents an independent proof that the reoxygenation-induced increment of gap formation is not caused by a further rise in cytosolic Ca\textsuperscript{2+}.

The contractile machinery of endothelial cells can be activated in a Ca\textsuperscript{2+}-dependent way through activation of myosin light chain kinase [8–10,22]. Contractile activation could be the underlying cause for the Ca\textsuperscript{2+} and energy-dependent cause for reoxygenation-induced gap formation. Therefore, we applied two chemically distinct inhibitors of MLC kinase, ML-7 and wortmannin, to the endothelial cells during reoxygenation. In these experiments we found the reoxygenation-induced gap formation blocked, indicating that contractile activation is indeed causing the gaps. Interestingly, variations of extracellular pH during reoxygenation did not change gap formation even though one might expect an influence of pH on contractile activation [23]. The likely explanation for this lack of pH\textsubscript{0} influence is that we investigated the contractile activation in the saturation range of Ca\textsuperscript{2+} overload where influences changing only Ca\textsuperscript{2+} sensitivity of the contractile machinery have no effect.

In summary, the study has revealed that aggravation of endothelial barrier failure represents a kind of reperfusion injury. The mechanism here identified depends on the energy-dependent activation of the endothelial contractile elements by causes intrinsic to endothelial cells. This mechanism affects endothelial barrier function within the first minutes of reoxygenation/reperfusion. In ischemic-reperfused myocardium other causes, extrinsic to endothelial cells can be expected to also contribute to endothelial barrier failure, e.g., release of oxygen radicals from activated leukocytes. Oxygen radicals have been shown to cause changes in endothelial cytoskeletal structures through Ca\textsuperscript{2+}-independent pathways and to promote intercellular gap formation [24].

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References


