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Stimulation of cGMP signalling protects coronary endothelium against reperfusion-induced intercellular gap formation

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Aims Ischaemia–reperfusion provokes barrier failure of the coronary microvasculature, impeding functional recovery of the heart during reperfusion. The aim of the present study was to investigate whether the stimulation of cGMP signalling by activation of soluble guanylyl cyclase (sGC) can reduce reperfusion-induced endothelial intercellular gap formation and to determine whether this is due to an influence on endothelial cytosolic Ca^{2+} homeostasis during reperfusion.

Methods and results Experiments were performed with cultured coronary endothelial monolayers and isolated saline-perfused rat hearts. HMR1766 (1 μmol/L) or DEAnonoate (0.5 μmol/L) were used to activate sGC. After exposure to simulated ischaemic conditions, reperfusion of endothelial cells led to a pronounced increase in cytosolic calcium levels and intercellular gaps. Stimulation of cGMP signalling during reperfusion increased Ca^{2+} sequestration in the endoplasmic reticulum (ER) and attenuated the reperfusion-induced increase in cytosolic [Ca^{2+}]. Phosphorylation of phospholamban was also increased, indicating a de-inhibition of the ER Ca^{2+} pump (SERCA). Reperfusion-induced intercellular gap formation was reduced. Reduction of myosin light chain phosphorylation indicated inactivation of the endothelial contractile machinery. Effects on cytsolic Ca^{2+} and gaps were abrogated by inhibition of cGMP-dependent protein kinase (PKG) with KT5823. In reperfused hearts, stimulation of cGMP signalling led to decreased oedema development.

Conclusion sGC/PKG activation during reperfusion reduces reperfusion-induced endothelial intercellular gap formation by attenuation of cytosolic calcium overload and reduction of contractile activation in endothelial cells. This mechanism protects the heart against reperfusion-induced oedema.

1. Introduction

Ischaemia–reperfusion disturbs the endothelial barrier function of the coronary system. The consequences are myocardial oedema and functional impairment of the myocardium.[1,2] Previous studies analysing the cellular mechanisms of endothelial barrier failure have revealed that this is due to opening of intercellular gaps, allowing the passage of fluid and macromolecules.[3–6] A major trigger for the process initiating these structural changes in barrier architecture is the rise of cytosolic Ca^{2+} in endothelial cells during ischaemia and reperfusion.[3,4,7,8] After prolonged ischaemia, reperfusion exerts an effect on its own with respect to endothelial cytosolic Ca^{2+} overload and intercellular gap formation.[4,6] This reperfusion-induced endothelial damage contributes to a specific aspect of reperfusion injury in the heart: myocardial oedema.

We showed previously that reperfusion-induced endothelial intercellular gap formation is due to an activation of the contractile machinery contained within the endothelial cells, as this, together with a loosening of cell–cell contacts, causes the opening of intercellular gaps.[4,6] During reperfusion, the endothelial contractile machinery is activated since Ca^{2+} overload and cellular resupply with ATP causes myosin light chain phosphorylation by activation of the Ca^{2+}-dependent MLC kinase pathway.[4] Strategies aiming either at a reduction of cytosolic Ca^{2+} overload or an inhibition of the endothelial contractile machinery can provide protection against reperfusion-induced intercellular gap formation.[4,6]

In the present study, we investigate the influence of cGMP signalling on reperfusion-induced endothelial Ca^{2+} overload, contractile activation, and intercellular gap formation. This investigation was motivated by reports that stimulation of cGMP signalling can reduce endothelial barrier dysfunction

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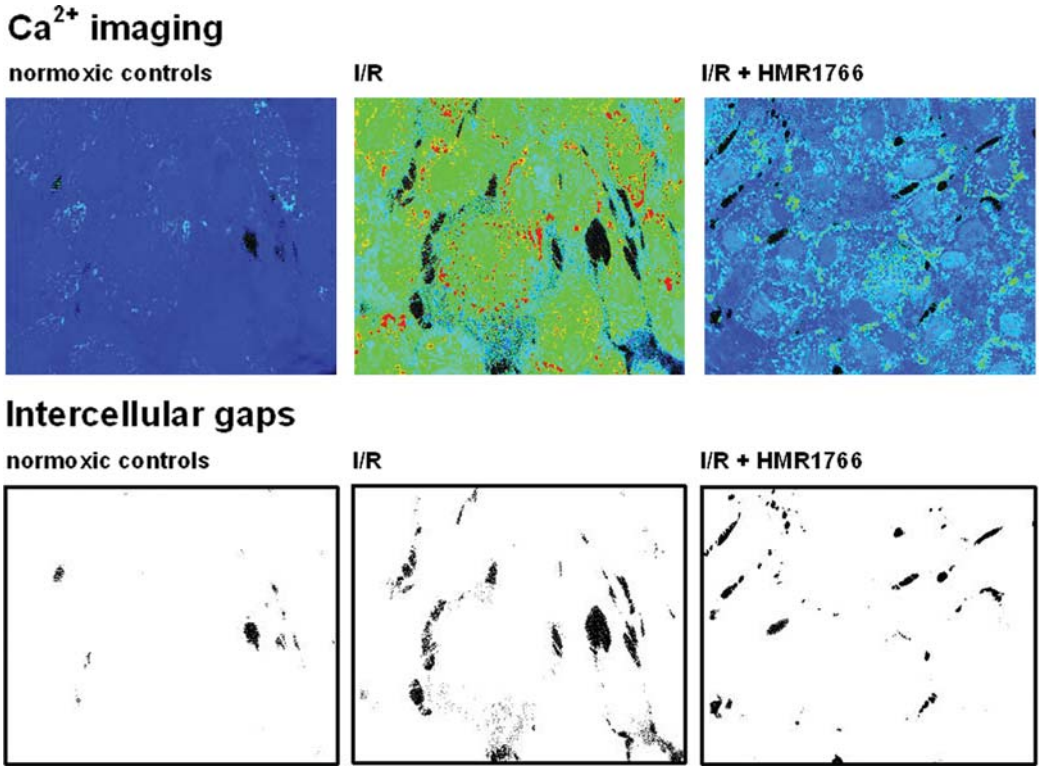

Figure 1 Representative micrographs of endothelial monolayers exposed to normoxic control conditions, 40 min simulated ischaemia plus 30 min reperfusion (I/R) with or without presence of HMR1766 during reperfusion. Top panel: fluorescence images of fura-2 loaded monolayers. Lower panel: binarized pictures from top panel showing all covered areas in white, gaps in black.

induced by oxidative stress, ionomycin, or thrombin.[9–13] The mechanistic analysis was performed on a culture model of coronary endothelium. Endothelial monolayers were exposed to simulated ischaemia and reperfusion. Two important influences of cGMP signalling on the mechanisms of endothelial barrier failure under reperfusion conditions were investigated in particular: First, its effect on the endoplasmic reticulum (ER), as this influences the disturbed cytosolic Ca^{2+} homeostasis, and second, its effect on the endothelial contractile machinery. Following the analysis on endothelial monolayers, the protective effects of cGMP signalling was also investigated on the intact coronary system of isolated perfused hearts.

cGMP signalling was stimulated either by HMR1766, a novel soluble guanylyl cyclase (sGC) activator preferentially targeting the NO-insensitive haem-oxidized form of sGC[14] or DEAnonoate, a conventional activator of sGC. To inhibit the cGMP-dependent protein kinase (PKG) the specific inhibitor KT5832 was used.

2. Methods

2.1 Cell culture

The 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).

Coronary endothelial cells were isolated from 200 to 250 g male Wistar rats and grown in culture as previously described.[4,15] Experiments were performed with confluent monolayers.

2.2 Experimental protocol

Endothelial cells cultured on glass cover slips were introduced into a perfusion chamber (0.5 mL filling volume) and were superfused at a flow rate of 0.5 mL/min with a modified HEPES buffer (composition in mmol/L: 140.0 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 2.5 glucose, and 25.0 HEPES), pH was adjusted to 7.4 or 6.4 at 30°C. Normoxic medium was equilibrated with air. In order to simulate ischaemia cells were exposed to an anoxic medium at $pH_0$6.4 for 40 min. The anoxic medium was glucose-free and equilibrated before and during experiments with 100% N_2. The medium was transferred into the perfusion chamber through gas-tight steel capillaries. PO_2 of anoxic medium at the chamber outlet was $<$1 mmHg as determined by a polarographic oxygen sensor. Reperfusion was performed for 30 min at $pH_0$7.4 with glucose.

HMR1766 (1 μmol/L) or DEAnonoate (0.5 μmol/L) were administrated during the whole reperfusion period. The inhibitor of the cGMP-dependent kinase, KT5823 (1 μmol/L), was added 10 min before and during reperfusion.

2.3 Ca^{2+} measurement and determination of intercellular gaps

Cytosolic concentration of Ca^{2+} was measured with the fluorescent indicator fura-2 AM as previously described.[4,16] Simultaneously to the measurement of the fura-2 ratio, intercellular gap formation was quantified by planimetrical analysis of the fluorescence images (*Figure 1*) as previously described.[4] The increase in reperfusion-induced gaps was related to the gap formation during anoxic perfusion, whereas normoxic values were set to 0% and gap formation after 40 min of anoxia was set to 100%.

2.4 Immunocytochemical analysis of MLC and PLB phosphorylation

Cells were treated as described earlier. Experiments were stopped at a given time point by the addition of ice cold methanol. After fixation cells were permeabilized 15 min with PBS containing 0.1% Tween and were subsequently blocked with PBS/BSA blocking solution for 1 h at room temperature. After blocking, cells were incubated with the primary antibody directed against the

phosphorylated MLC (1:500) or phospholamban (1:500) for 1 h at room temperature, followed by a washing step with PBS. Cells were then incubated with a biotin conjugated anti-mouse IgG secondary antibody (1:20 000) for 30 min followed by extensive washing steps with PBS. Finally cells were incubated with Texas Red conjugated Streptavidin (1:800) overnight after which they were washed for 30 min with PBS, mounted on microscope slides and fluorescence was detected. The excitation wavelength for Texas Red was 594 nm.

2.5 Myocardial water content

Hearts from 200 to 250 g male Wistar rats were mounted immediately after isolation on a Langendorff perfusion system in a temperature-controlled chamber (37°C), as previously described.[6] Briefly, during normoxic perfusion at constant flow (7 mL/min) with an oxygenated, cardioplegic, hyperkalemic saline medium [composition in mmol/L: 122.7 NaCl, 24.0 $NaHCO_3$, 20.0 KCl, 0.4 KH_2PO_4, 1.0 $MgSO_4$, 1.8 $CaCl_2$, 5.0 glucose, pH 7.4; gassed with 95% O_2 (vol/vol)/5% CO_2(vol/vol)], the chamber was flushed with humidified air, and during anoxia (no-flow), with humidified 100% N_2. After 40 min of no-flow ischaemia, hearts were again resupplied with oxygen by reperfusion of a cardioplegic, hyperkalemic saline solution for 40 more minutes. For reperfusion, constant flow conditions (7 mL/min) were again chosen. Under these conditions it can be assumed that capillary hydrostatic pressure remains largely unaffected by vasomotor effects and hence water filtration is a function of endothelial permeability (Starling's Law). This is because vasomotor effects are predominantly on the pre-capillary level and not on the capillary or post-capillary level, i.e. they do not alter capillary resistance. According to Ohm's Law capillary pressure is constant when capillary flow and resistance are constant. Once large water shifts into the interstitium have occurred, capillary resistance increases. Then, changes in water shifts underestimate the changes in permeability, a conservative error for the aims of this study.

HMR1766 (1 μmol/L) or DEAnonoate (0.5 μmol/L) with or without KT5823 (1 μmol/L) was added to the perfusion medium 1 min before the onset of cardioplegic reperfusion. It remained in the perfusion medium during the entire period of reperfusion. At the end of each experiment wet weight and after 24 h of drying at 70°C dry weight of the perfused rat hearts were measured. Cardioplegic conditions were chosen to exclude indirect effects on cardiac water content through changes in contractile function.

2.6 Materials

HMR1766 was from Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany); Falcon plastic tissue culture dishes were from Becton-Dickinson (Heidelberg, Germany); fura-2 AM was from Molecular Probes (Eugene, OR); ionomycin from Calbiochem (Bad Soden Germany); FCS, medium 199, penicillin-streptomycin, and trypsin-EDTA were from GIBCO Life technologies (Eggenstein, Germany). All other chemicals were of the best available quality, usually analytic grade.

2.7 Statistical analysis

Values are expressed as means ± SEM from *n* experiments using independent 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

3.1 Effects of HMR 1766 and DEAnonoate on intracellular Ca^{2+} and gap formation during anoxia and reperfusion

Endothelial monolayers were submitted to simulated ischaemia and reperfusion. The time courses of cytosolic Ca^{2+} and intercellular gap formation were analysed. First, we investigated the influence of the activator of sGC, HMR1766. *Figure 2* shows a representative trace of the fura-2 ratio under control conditions and in the presence of HMR1766 (1 μmol/L), which was added with the onset of reoxygenation. As shown before, cytosolic Ca^{2+} rises in simulated ischaemia in a biphasic manner. Initially, there is a transient Ca^{2+} increase, due to Ca^{2+} release from the ER. This phase is followed by a progressive and sustained Ca^{2+} rise, due to Ca^{2+} influx across the plasmalemma. Simulated reperfusion conditions caused a further increase in cytosolic Ca^{2+}.[4,6–8] When HMR1766 was present during reperfusion, however Ca^{2+} fell below the end-ischaemic value (*Figures 1* and *2*). Ischaemic conditions lead to the opening of intercellular gaps, and this gap formation was accelerated by reperfusion, shown by original recordings in *Figures 1* and *3*. HMR1766 (1 μmol/L) prevented the reperfusion-induced augmentation of gap formation. Effects of HMR1766 on Ca^{2+} rise and gap formation were dose-dependent as determined in a concentration range from 0.1 to 1 μmol/L (data not shown). As the maximum effect was observed at a concentration of 1 μmol/L HMR1766 was used in that concentration in all following experiments.

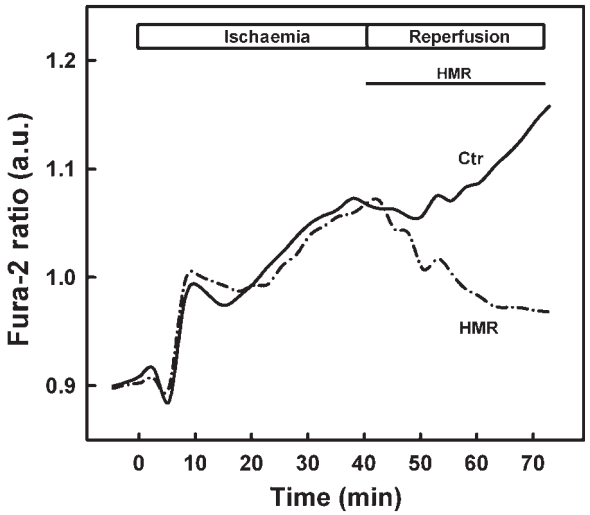

Figure 2 Representative traces of cytosolic Ca^{2+} concentration (Fura-2 ratio) of coronary endothelial monolayers during simulated ischaemia and reperfusion in the presence or absence of the sGC activator HMR1766 (1 μmol/L).

The effects of HMR1766 on Ca^{2+} and gap formation during reperfusion were compared with that of the NO-donor DEAnonoate (0.5 μmol/L). *Figures 4* and *5* show that both compounds reduced the reperfusion-induced rise in cytosolic Ca^{2+} and gap formation. The effects of these compounds were abolished if these were applied together with KT5823 (1 μmol/L), an inhibitor of cGMP-dependent kinase (PKG). This indicates that these effects are mediated through an activation of PKG.

3.2 Effects of HMR1766 and DEAnonoate on endoplasmic reticulum function

In endothelial cells, the ER plays an important role in the regulation of cytosolic Ca^{2+} homeostasis. We tested the hypothesis whether the reduction of cytosolic Ca^{2+} achieved by stimulation of cGMP signalling during reperfusion is due to enhanced Ca^{2+} sequestration into the ER. For this purpose, the pool of thapsigargin-releasable Ca^{2+} at the

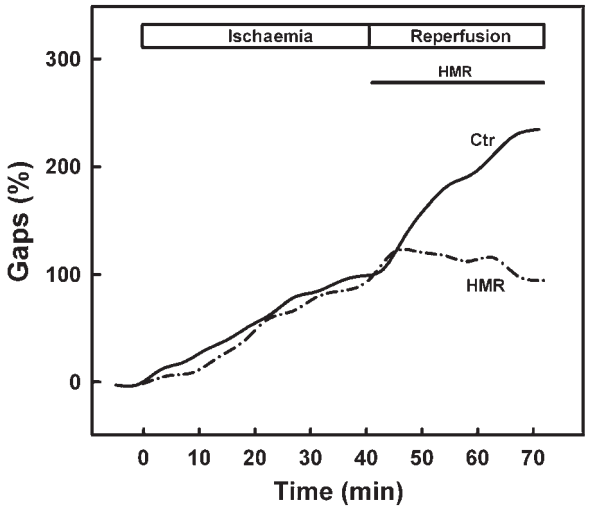

Figure 3 Representative traces of intercellular gap formation of coronary endothelial monolayers during simulated ischaemia and reperfusion in the presence or absence of the sGC activator HMR1766 (1 μmol/L).

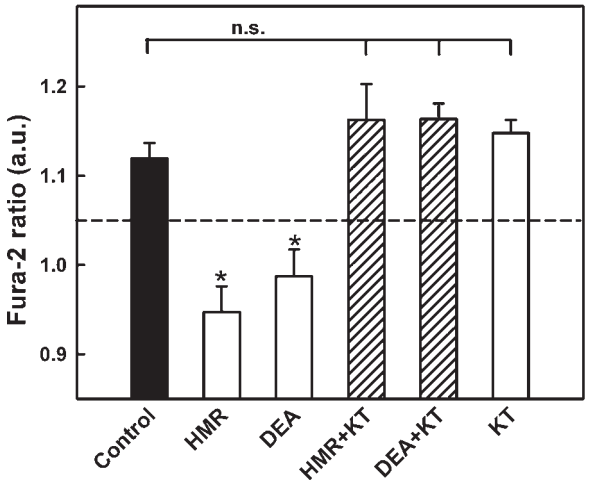

Figure 4 The effects of guanylyl cyclase activation in the absence or presence of the cGMP-dependent protein kinase inhibitor KT5823 (KT) on reperfusion induced increase of cytosolic Ca^{2+} concentration after 30 min of reperfusion. Dashed line represents values of cytosolic Ca^{2+} after 40 min of simulated ischaemia. Data are means ± SEM of $n = 5$ separate experiments with independent cell preparations. *$P < 0.05$ vs. control; n.s., not significantly different.

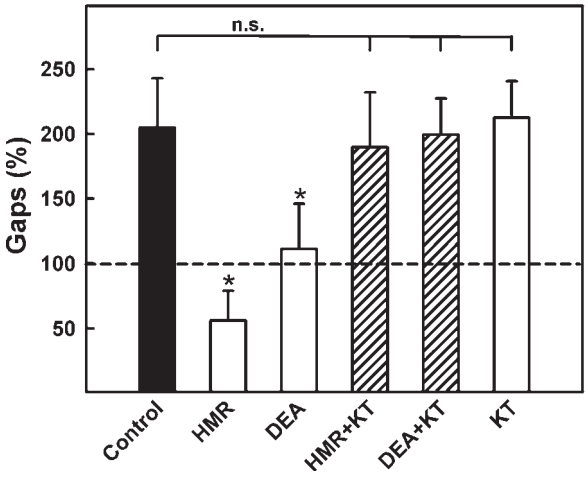

Figure 5 The effects of guanylyl cyclase activation in the absence or presence of the cGMP-dependent protein kinase inhibitor KT5823 (KT) on reperfusion induced increase of intercellular gap formation after 30 min of reperfusion. Dashed line represents values of intercellular gap formation after 40 min of simulated ischaemia. Data are means ± SEM of $n = 5$ separate experiments with independent cell preparations. *$P < 0.05$ vs. control; n.s., not significantly different.

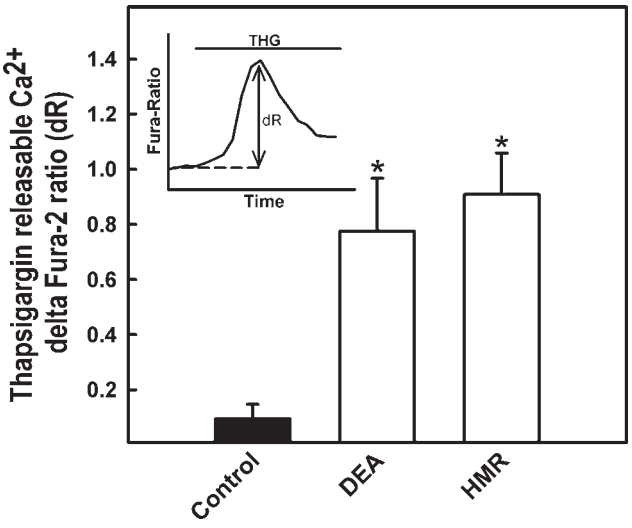

Figure 6 Thapsigargin-releaseable Ca^{2+} in endothelial cells after different reperfusion conditions, i.e. in the absence (Control) or presence of guanylyl cyclase activators (HMR,DEA). Thapsigargin (100 nmol/L) was added after the reperfusion period and calcium measurements were continued to record the subsequent cytosolic calcium rise. Data are means ± SEM of $n = 5$ separate experiments with independent cell preparations. *$P < 0.05$ vs. control.

end of the 30 min reperfusion period was determined by the administration of 100 nM thapsigargin and recording of the subsequent rise of fura-2 fluorescence (*Figure 6*). In the presence of either HMR1766 or DEAnonoate this pool was significantly enlarged. This indicates that reduction of cytosolic Ca^{2+} mediated by stimulation of cGMP signalling is indeed due to an enhanced sequestration of Ca^{2+} into the ER.

Since ER Ca^{2+} loading is controlled by the ER Ca^{2+} ATPase (SERCA) and this in turn by the phosphorylation state of its regulating protein, phospholamban,[17] we tested whether stimulation of cGMP signalling is able to increase phospholamban phosphorylation under reperfusion conditions. As shown in *Figure 7*, application of HMR1766 or DEAnonoate during reoxygenation increased the phosphorylation of phospholamban. This effect was abrogated, however, in the co-presence of the PKG inhibitor KT5823.

3.3 Effects of HMR1766 and DEAnonoate on MLC phosphorylation

Since the activation of the contractile machinery of endothelial cells depends on the phosphorylation state of the light chains of myosin (MLC),[18,19] we analysed MLC phosphorylation in reperfused endothelial cells. As shown in *Figure 8*, MLC phosphorylation was significantly increased under control reperfusion conditions. This increase in MLC phosphorylation in reperfused endothelial cells was significantly reduced by both tested compounds (*Figure 8*).

3.4 Effects of HMR1766 and DEAnonoate on myocardial water content

To verify whether the activation of cGMP signalling can stabilize the endothelial barrier *in situ*, additional experiments

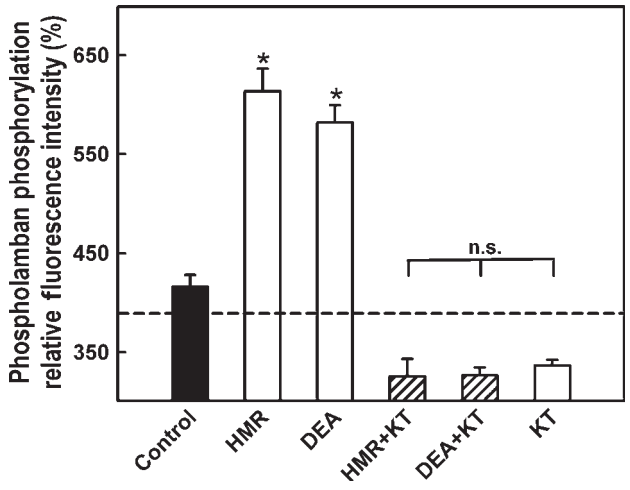

Figure 7 The effect of two different guanylyl cyclase activators (HMR,DEA) in the absence or presence of the cGMP-dependent protein kinase inhibitor KT5823 (KT) on phospholamban phosphorylation after simulated ischaemia and reperfusion. Dashed line represents fluorescence intensity after 40 min of simulated ischaemia, whereas normoxic values were set to 100%. Data are means ± SEM of $n = 5$ separate experiments with independent cell preparations. *$P < 0.05$ vs. control; n.s., not significantly different.

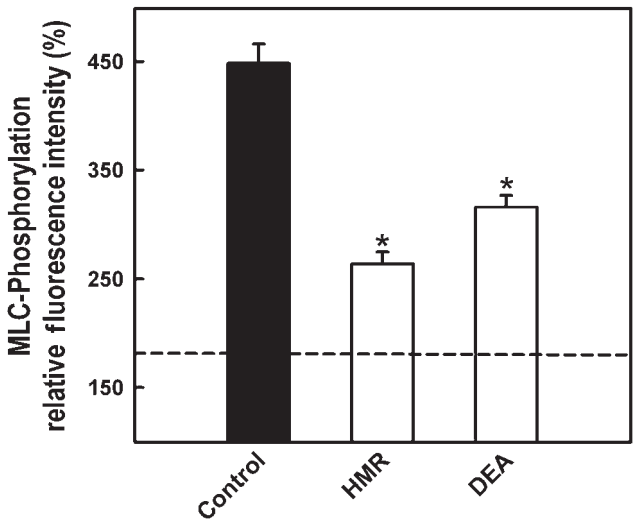

Figure 8 The effect of two different guanylyl cyclase activators on MLC phosphorylation after simulated ischaemia and reperfusion. Dashed line represents fluorescence intensity after 40 min of simulated ischaemia, whereas normoxic values were set to 100%. Data are means ± SEM of $n = 5$ separate experiments with independent cell preparations. *$P < 0.05$ vs. control.

were performed on Langendorff-perfused isolated hearts. Myocardial water content was determined as parameter for tissue oedema. Cardioplegic conditions were chosen to exclude indirect effects on cardiac water content through changes in contractile function. Hearts were exposed for 40 min to no-flow ischaemia and then reperfused for another 40 min. Reperfusion of ischaemic hearts caused an increase in myocardial water content from 5.98 ± 0.47 mL H_2O/g dry weight at the onset of reperfusion to 7.90 ± 0.23 mL H_2O/g dry weight after 40 min of reperfusion. If HMR1766 or DEA-nonoate was added during reperfusion, the reperfusion-induced increase in myocardial water was markedly reduced. This effect was abrogated, again, in the co-presence of the PKG inhibitor KT5823 (*Figure 9*). The various treatments caused moderate changes in aortic perfusion pressure during the reperfusion period. For the 40 min of reperfusion, the inflow pressure changed from an average of 73.7 ± 6.0 cmH_2O under control conditions to 57.1* ± 3.5 cmH_2O (HMR) or 61.3* ± 5.5 cmH_2O (DEA), respectively. Changes in perfusion pressure during application of KT5823, alone or in combination with sGC activation, were not significantly different from control conditions ($n = 6$–8; * < 0.05 vs. control).

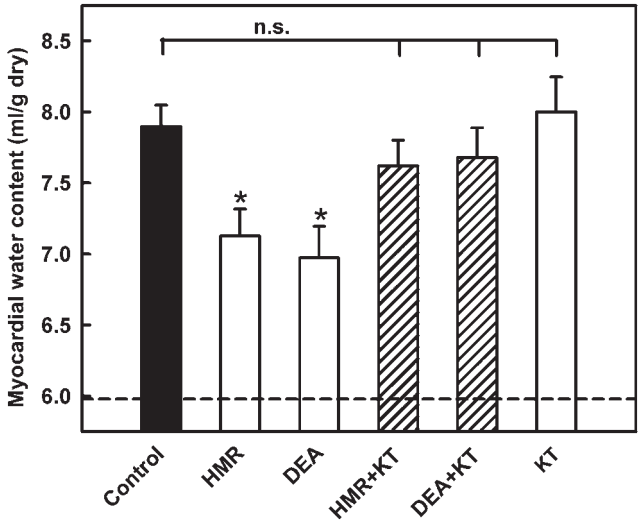

Figure 9 Myocardial water content of isolated, saline perfused hearts after conditions of simulated ischaemia and reperfusion. Hearts were exposed to 40 min of no-flow ischaemia followed by 40 min of reperfusion (Control). Dashed line represents values after 40 min of no-flow ischaemia. Guanylyl cyclase activators (HMR,DEA) were added during reperfusion in the absence or presence of the cGMP-dependent protein kinase inhibitor KT5823 (KT). Data are means ± SEM of $n \geq 5$ separate experiments. *$P < 0.05$ vs. control.

4. Discussion

The principal finding of this study is that stimulation of cGMP signalling protects the coronary endothelium against reperfusion-induced barrier failure. Analysis at the cellular level showed that this cGMP-mediated protection is due to enhanced sequestration of cytosolic Ca^{2+} into the ER and reduction of MLC phosphorylation, resulting in a slow-down of contractile activation in endothelial cells and hence attenuation of intercellular gap formation.

The cell-culture model used in the present study, consisting of endothelial cells exposed to conditions of simulated ischaemia and reperfusion, was described in detail previously.[4,6,7] The present study was focused on changes induced by reperfusion conditions. When occurring after prolonged ischaemia, reperfusion aggravates the disturbance of cytosolic Ca^{2+} control in endothelial cells. This leads to ongoing or even aggravated disturbance of endothelial cell functions which represent a part of the reperfusion injury that can be observed in reperfused myocardium. Reperfusion injury is elicited by the specific circumstances of reperfusion in a previously ischaemic cell. As we have shown before, reperfusion injury to the endothelial barrier can be elicited by Ca^{2+}-overload-induced activation of the endothelial contractile machinery. It was here shown that a partial Ca^{2+} recovery can be achieved during the early phase of reperfusion even after prolonged ischaemia, when stimuli of cGMP signalling are applied upon the onset of reperfusion. Stimuli here investigated

were activators of sGC, namely HMR1766 or DEAnonoate. Their effects could be abolished by a PKG inhibitor, indicating that this protein kinase is involved in the signalling.

The decline of cytosolic Ca^{2+} overload achieved in reperfused endothelial cells in the presence of the stimuli of cGMP signalling corresponded to increased storage of Ca^{2+} in the ER, indicating a causal relationship. Experimentally, the filling of the ER was evaluated by comparing the thapsigargin-releasable Ca^{2+} pools between the different conditions. An increase in Ca^{2+} sequestration in the ER can be due to a stimulation of the ER Ca^{2+} pump (SERCA). Since SERCA activity is enhanced by phosphorylation of its regulating protein, phospholamban, the phosphorylation state of this protein can be analysed as an indicator for SERCA activity in intact cells. Results of this study show that the investigated cGMP stimuli caused an upregulation of phospholamban phosphorylation in reperfused endothelial cells. Abrogation of this phosphorylation of phospholamban by a PKG inhibitor indicates that it is down-stream of PKG activation. It remains open whether phospholamban is a direct substrate of PKG in these cells.

Stimuli of cGMP signalling also reduced gap-formation in reperfused endothelial cells. As shown before, the formation of intercellular gaps is dependent on the activation of the endothelial contractile machinery[4] and this in turn is indirectly controlled by the cytosolic Ca^{2+} level. The main regulatory elements in the contractile machinery are the myosin light chains, controlled by the Ca^{2+}-dependent MLC kinase. Reperfusion conditions bring together a high cytosolic Ca^{2+} load and the ATP required for the MLC kinase and actomyosin ATPase. These conditions can thereby initiate a retraction of cells from each other, thus opening intercellular gaps.[4] The results of this study show that in the presence of stimuli of cGMP MLC phosphorylation is indeed reduced in reperfused endothelial cells. It is possible that this is not entirely due to reduced activation of the Ca^{2+}-dependent MLC kinase, but also to enhanced activation of MLC phosphatase, as shown for PKG in smooth muscle cells.

Even though the cellular model of ischaemia and reperfusion used in this study does not reflect the whole scenario for ischaemia/reperfusion *in vivo*, it has proved to be a valuable tool for investigating simultaneous changes in cytosolic ion homeostasis and cell morphology. Its use in the present study reveals for the first time that activation of cGMP signalling in endothelial cells can interfere with detrimental reperfusion effects on endothelial barrier function. To verify that these effects are not restricted to this simulated *in vitro* culture model, the stimuli of cGMP signalling were also applied to the intact coronary bed *in situ*, using an isolated, saline perfused heart. In this model, we found a significant reduction of reperfusion induced increase of myocardial water content if an activator of sGC, i.e. HMR1766 or DEAnonoate, was applied during reperfusion. As argued in the Methods section, under constant flow conditions changes in water content do indeed reflect changes in permeability. Briefly, this is because the vasomotory effects of the various agents affect pre-capillary and not capillary flow resistance and hence, according to Ohm's Law, do not affect capillary hydrostatic filtration pressure.

The comparison of gap formation in the cell culture model and oedema development in the intact, but saline-perfused heart should not be over-stretched. First, cultured coronary cells are not the same as coronary cells *in situ*. Second, the perfusion conditions are quite different, with a much higher ratio of volume flux to cell mass in the culture system. Third, tissue water uptake is only a coarse parameter for endothelial permeability which does not differentiate between the extravascular subcompartments filling up with water. But in spite of these limitations and the relative differences seen between these two experimental models, the results from both speak the same general language in respect to the cGMP-mediated protection.

Interestingly, the protective effects of stimuli of cGMP signalling under reperfusion conditions are not confined to the endothelial cell type in the heart. We showed previously that cGMP signalling enhances Ca^{2+} sequestration by SERCA also in cardiomyocytes under reperfusion, preventing them from hypercontracture, a characteristic feature of reperfusion injury on the cardiomyocyte level.[20] The protective effects of cGMP signalling on either cell type have the potential to be exploitable for the protection of the reperfused heart *in vivo*.

In the present study, we used different approaches to stimulate cGMP signalling in endothelial cells, but they showed comparable effects on Ca^{2+} homeostasis, MLC phosphorylation, and hence gap formation. Among the stimuli used, the NO donor DEAnonoate may be the least suitable for a therapeutic application. This is because NO can exert deleterious effects by its own due to its radical nature[21] either directly, or through generation of peroxynitrite.[22] This problem is avoided by agents activating the sGC directly such as HMR1766.

In conclusion, activation of the cGMP signalling pathway through stimulation of sGC effectively protects the coronary endothelium against reperfusion-induced barrier failure, in cultured coronary endothelium as well as in the coronary system *in situ*.

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Conflict of interest: none declared.

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