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Lithium prevents early cytosolic calcium increase and secondary injurious calcium overload in glycolytically inhibited endothelial cells

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ABSTRACT

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) is a central signalling element for the maintenance of endothelial barrier function. Under physiological conditions, it is controlled within narrow limits. Metabolic inhibition during ischemia/reperfusion, however, induces $[Ca^{2+}]_i$ overload, which results in barrier failure. In a model of cultured porcine aortic endothelial monolayers (EC), we addressed the question of whether $[Ca^{2+}]_i$ overload can be prevented by lithium treatment. $[Ca^{2+}]_i$ and ATP were analysed using Fura-2 and HPLC, respectively. The combined inhibition of glycolytic and mitochondrial ATP synthesis by 2-desoxy-D-glucose (5 mM; 2-DG) plus sodium cyanide (5 mM; NaCN) caused a significant decrease in cellular ATP content (14 ± 1 nmol/mg protein vs. 18 ± 1 nmol/mg protein in the control, $n = 6$ culture dishes, $P < 0.05$), an increase in $[Ca^{2+}]_i$ (278 ± 24 nM vs. 71 ± 2 nM in the control, $n = 60$ cells, $P < 0.05$), and the formation of gaps between adjacent EC. These observations indicate that there is impaired barrier function at an early state of metabolic inhibition. Glycolytic inhibition alone by 10 mM 2-DG led to a similar decrease in ATP content (14 ± 2 nmol/mg vs. 18 ± 1 nmol/mg in the control, $P < 0.05$) with a delay of 5 min. The $[Ca^{2+}]_i$ response of EC was biphasic with a peak after 1 min (183 ± 6 nM vs. 71 ± 1 nM, $n = 60$ cells, $P < 0.05$) followed by a sustained increase in $[Ca^{2+}]_i$. A 24-h pre-treatment with 10 mM of lithium chloride before the inhibition of ATP synthesis abolished both phases of the 2-DG-induced $[Ca^{2+}]_i$ increase. This effect was not observed when lithium chloride was added simultaneously with 2-DG.

We conclude that lithium chloride abolishes the injurious $[Ca^{2+}]_i$ overload in EC and that this most likely occurs by preventing inositol 3-phosphate-sensitive Ca^{2+}-release from the endoplasmic reticulum. Though further research is needed, these findings provide a novel option for therapeutic strategies to protect the endothelium against imminent barrier failure.

1. Introduction

Failure of endothelial barrier function occurs immediately in response to ischemia [12,19]. Under these conditions, when cellular adenosine-5′-triphosphate (ATP) decreases, the accumulation of cytosolic free calcium concentration $[Ca^{2+}]_i$ triggers a variety of pivotal physiological and/or injurious pathophysiologic responses in endothelial cells [19,27]. It has been shown that cultured endothelial cells (EC) from different vascular regions, e.g., porcine aorta [24] or rat coronary microvessels [12] as well as EC of the intact intima of aortic vessel segments ex vivo [23], respond to manoeuvres that block endothelial energy, such as deep hypoxia, ischemia or direct metabolic inhibition, with a distinct biphasic increase of $[Ca^{2+}]_i$ [19,23]. In its initial phase, this $[Ca^{2+}]_i$ increase is due to an activation of the D-myo-inositol 3-phosphate (IP_3) -sensitive Ca^{2+}-release mechanism of the endoplasmatic reticulum (ER). The local decline in ATP seems to sensitise this mechanism to IP_3, or it may lead to a rapid up-regulation of IP_3, resulting in Ca^{2+} discharge from the ER [9,10,25]. The initial $[Ca^{2+}]_i$ increase can be followed by a secondary influx of Ca^{2+} from the extracellular space via store-operated or calcium-gated Ca^{2+} channels of the plasma membrane [19,27]. The increase in $[Ca^{2+}]_i$ activates the endothelial

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actomyosin-based contractile machinery, triggering cell retraction, and propagates the development of gaps between adjacent endothelial cells, where cell adhesion structures have been disturbed [7,19,23]. Such endothelial barrier failure may lead to a vasogenic and interstitial oedema with unfavourable consequences in nearly all vascular territories or organs.

On the cellular level, endothelial Ca^{2+} homeostasis is regained via Ca^{2+} reuptake into the ER or outward transport via the plasma membrane by Ca^{2+}-ATPases [23,29]. Therefore, $[Ca^{2+}]_i$ overload, and thus vasogenic oedema, may be reversible. In severe and prolonged ischemia, however, the recovery of Ca^{2+} homeostasis can be insufficient. Under these conditions, endothelial cells may be irreversibly damaged, especially when $[Ca^{2+}]_i$ overload persists [15] and triggers disintegration of the endothelial cytoskeleton and intercellular junctions [14,22]. At present, the therapeutic strategies to prevent this injurious $[Ca^{2+}]_i$ overload and the consequent irreversible, fatal end-stage endothelial barrier failure are largely unknown.

In this regard, lithium seems to be a potential candidate for $[Ca^{2+}]_i$ overload prevention. Lithium is a common drug for the prophylaxis and treatment of psychiatric (bipolar affective) disorders [5] and interacts with the inositol metabolism of various cell types [1,3]. It is known to block the "recycling" of phosphatidylinositol-4,5-bisphosphate (PIP_2) to IP_3. The resulting loss of IP_3 influences the release of Ca^{2+} from the ER [2]. However, less is known about the influence of lithium on the endothelial $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Cell culture

EC from the porcine aorta were isolated and cultured as previously described [14]. Confluent cultures of primary EC were seeded at a density of 70,000 cells/cm^2 on 25-mm round glass coverslips for determination of $[Ca^{2+}]_i$ or 30-mm culture dishes. Experiments were performed with confluent endothelial monolayers four days after seeding. This study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Experimental protocol

Endothelial monolayers were incubated at 37 °C in a HEPES-buffered solution (composition at pH 7.4: 25 mM HEPES, 125 mM NaCl, 1.0 mM $CaCl_2$, 2.6 mM KCl, 1.2 mM $MgCl_2$, and 1.2 mM KH_2PO_4), supplemented with 2% (vol/vol) heat-inactivated newborne calf serum. The cells were treated identically to allow a comparison between alterations in $[Ca^{2+}]_i$ and the corresponding ATP content. In the first set of experiments, metabolic inhibition was achieved by the addition of 2-deoxy-D-glucose (2-DG) plus sodium cyanide (NaCN) or by 2-DG alone. Lithium chloride was added 24 h before metabolic inhibition to deplete EC of IP_3. In another set of experiments, lithium chloride was simultaneously added with 2-DG. Stock solutions of the substances were prepared in the HEPES-buffered solution. Appropriate volumes of the solutions were added to the EC, and identical additions were made in all respective control experiments.

2.3. Analysis of $[Ca^{2+}]_i$

The cytosolic free Ca^{2+} concentration of EC was determined with the fluorescent Ca^{2+} fura-2-acetoxymethyl ester (Fura-2) in a life cell imaging system (TILL Photonics, Martinsried, Germany) as previously described [20].

2.4. Analysis of cellular ATP

To analyse the cellular ATP content, the incubation medium was aspirated, and ice-cold perchloric acid (0.6 M $HClO_4$) was added to immediately terminate the incubation. The extract was neutralised, and the amount of ATP was determined by high-performance liquid chromatography as previously described [13]. The ATP contents are expressed in relation to the cellular protein contents (nmol ATP/mg protein). The cellular protein contents were determined according to Bradford [4] using bovine serum albumin as the standard.

2.5. Statistical analysis

All values are expressed as the mean ± S.E. of n experiments using three or more independent EC monolayer preparations. Statistical analysis was performed using a one-way ANOVA in conjunction with the Student–Newman–Keuls test for post hoc analysis. Between-group analysis was performed. $P < 0.05$ was considered significant.

3. Results

Confluent cultures of endothelial cells from porcine aortas responded instantaneously to metabolic inhibition by 5 mM 2-DG plus 5 mM NaCN with an increase in $[Ca^{2+}]_i$ and barrier failure. As shown in Fig. 1A and B, $[Ca^{2+}]_i$ increased significantly from a basal level of 71 ± 2 nM to 278 ± 24 nM, and gaps formed between adjacent EC immediately after the blockade of glycolytic and mitochondrial ATP synthesis. Both effects were further enhanced by ongoing metabolic inhibition.

To prove whether this manoeuvre led to an inhibition of ATP synthesis, the cellular ATP content was determined in EC treated under identical conditions. As shown in Fig. 2A, the cellular ATP content significantly declined from a basal level of 18 ± 1 nmol/mg protein to 14 ± 1 nmol/mg protein after 1 min and to 2.7 ± 1 nmol/mg protein after 15 min of metabolic inhibition.

Inhibition of the glycolytic energy production by 10 mM 2-DG alone also caused a reduction of the cellular ATP content, but this was much slower. As shown in Fig. 2B, the ATP content was reduced from 18 ± 2 nmol/mg protein to 14 ± 2 nmol/mg protein after 5 min and further declined to 13 ± 2 nm/mg protein after 15 min.

Nevertheless, glycolytic inhibition by 2-DG alone caused a biphasic increase in $[Ca^{2+}]_i$, as shown by a representative recording of the Fura-2 fluorescence ratio in Fig. 3A. A 24-h pre-incubation with lithium (10 mM) was used to deplete the IP_3 in EC and, thereby, potentially block the IP_3-sensitive Ca^{2+} release mechanism of the ER. After this manoeuvre, the 2-DG-induced increase in the fluorescence ratio was abolished. It is worth mentioning that there was neither an initial nor a secondary sustained increase in the fluorescence ratio (Fig. 3A).

The biphasic 2-DG-induced response was analysed in more detail. For the two time points of 1 min (initial phase) and 15 min (secondary phase) after 2-DG addition, the Fura-2 fluorescence ratio was quantified, and the effect of 2-DG on endothelial $[Ca^{2+}]_i$ was determined under three different conditions: in the presence of 2-DG alone, after simultaneous addition of 2-DG plus lithium chloride (short-term effects of lithium), and after the addition of 2-DG following treatment with lithium chloride for 24 h (long-term effects of lithium). As shown in Fig. 3B, 2-DG caused an increase of $[Ca^{2+}]_i$ from a basal level of 72 ± 4 nM to 181 ± 8 nM after 1 min and further to 236 ± 9 nM after 15 min. The simultaneous addition of 10 mM of 2-DG plus 10 mM of lithium chloride caused almost the same increase in $[Ca^{2+}]_i$ (1 min: 183 ± 7 nM; 15 min:

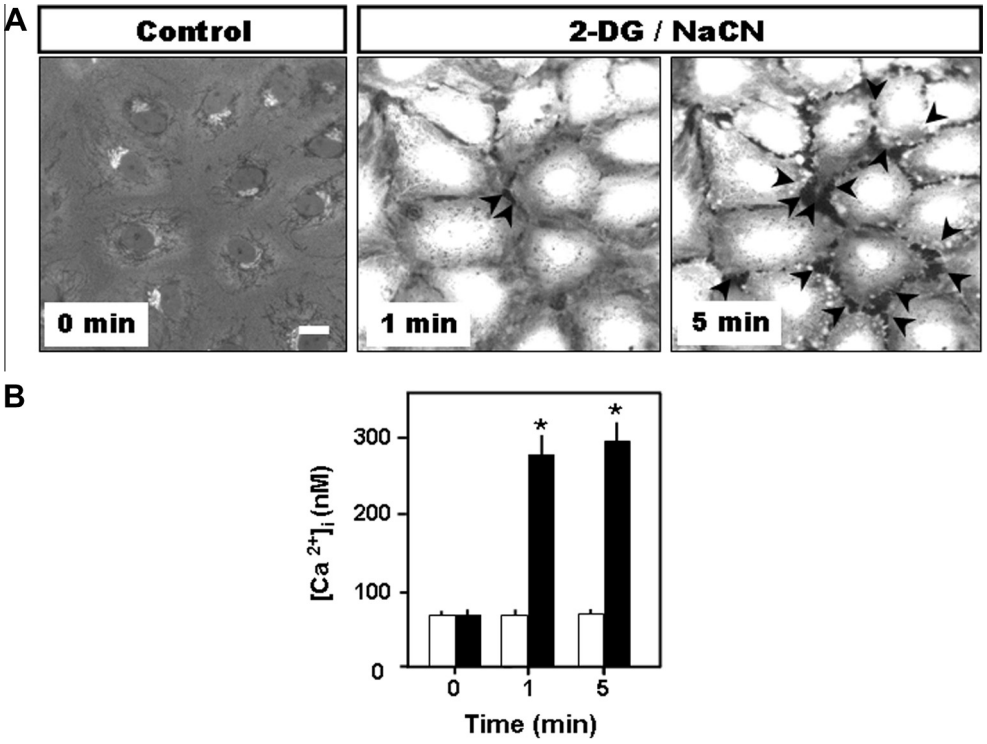

Fig. 1. Effect of metabolic inhibition on $[Ca^{2+}]_i$ and interendothelial gap formation on monolayers of porcine aortic endothelial cells. (A) Representative microscopic images of endothelial monolayers loaded with the Ca^{2+} indicator Fura-2 recorded by a Ca^{2+}-imaging system. Images are of the control cells (time point zero) at 1 or 5 min after the combined addition of 2-deoxy-D-glucose (2-DG, 5 mM) plus sodium cyanide (NaCN, 5 mM) to block glycolytic and mitochondrial energy production. At time zero, the $[Ca^{2+}]_i$ is low, as indicated by the overall dark appearance of the cell monolayer in the Fura-2 image. There are no gaps between the endothelial cells. In contrast, 1 min after the addition of 2-DG/NaCN, $[Ca^{2+}]_i$ is increased, as indicated by the bright image and gap formation (arrow heads). After 5 min of metabolic inhibition, gap formation is further enhanced. Scale bar represents 10 μm. (B) Quantitative analyses of the $[Ca^{2+}]_i$ records as shown in (A). (Open bars) $[Ca^{2+}]i$ represent untreated control cells; (closed bars) $[Ca^{2+}]_i$ represent cells exposed to 5 mM 2-DG plus 5 mM NaCN. Upon metabolic inhibition, endothelial cells respond with a biphasic increase in $[Ca^{2+}]_i$ within 15 min. Data are the mean ± SE of $n = 60$ EC, $^*P < 0.05$ vs. control.

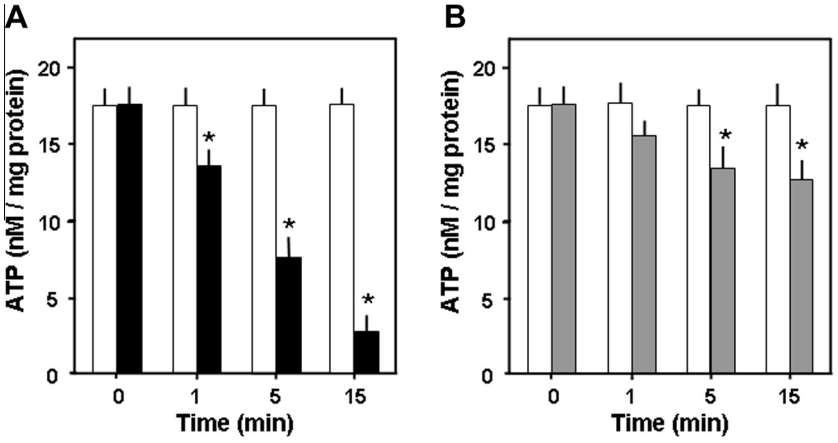

Fig. 2. Effect of metabolic inhibition on endothelial ATP content. (A) Endothelial cells were exposed to 5 mM of 2-deoxy-D-glucose plus 5 mM of sodium cyanide (closed bars); (control, open bars). ATP immediately starts to decline and is significantly reduced after 1 min. (B) Cells exposed to only 10 mM of 2-DG (closed bars); (control, open bars). Inhibition of glycolytic energy production alone also induced a reduction in the cellular ATP content. However, ATP declined more slowly and was significantly reduced after 5 min. Data are the mean ± SE of $n = 6$ culture dishes of three independent cell preparations, $^*P < 0.05$ vs. control.

232 ± 8 nM), indicating that lithium chloride does not have an acute effect on the 2-DG-induced increase in $[Ca^{2+}]_i$. In contrast, 2-DG failed to increase $[Ca^{2+}]_i$ in EC pre-treated with 10 mM lithium for 24 h (Fig. 3B).

4. Discussion

The present study addresses the question of whether long-term exposure to lithium can prevent the deleterious Ca^{2+}-overload induced by metabolic inhibition in endothelial cells. Deep hypoxia, ischemia or manoeuvres directly blocking energy production induces a biphasic increase in endothelial $[Ca^{2+}]_i$. It has been shown that this -$[Ca^{2+}]_i$ increase includes two main components, an IP_3-sensitive Ca^{2+}-release mechanism from the ER that triggers the subsequent sustained influx of Ca^{2+} via the plasma membrane [19,23]. One major aim of this study was to inhibit the IP_3-sensitive Ca^{2+}-release mechanism of the ER by long-term pre-treatment with lithium to deplete endothelial cells of inositol phosphates. Moreover, we hypothesise that targeted inhibition of the initial

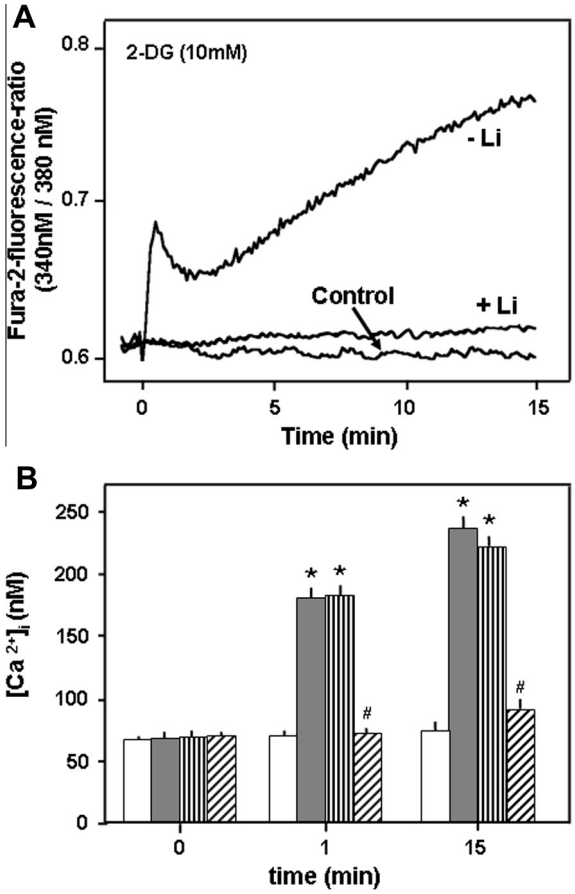

Fig. 3. Effect of 2-deoxy-D-glucose (2-DG, 10 mM) on the Fura-2 fluorescence ratio of porcine EC with and without pre-incubation with lithium (10 mM) for 24 h. (A) A representative experiment (continuous monitoring of the Fura-2 fluorescence ratio) after the addition of 2-DG is shown, demonstrating a typical biphasic increase of the fluorescence ratio with two maxima at approximately 1 and 15 min on the one side and a nearly abolished fluorescence ratio increase after a 24-h pre-incubation with lithium on the other side. The effects of lithium are not observed in the control. (B) Effect of lithium chloride on 2-DG-induced increase of $[Ca^{2+}]_i$. (□) non-treated control cells; (▨) cells exposed to 10 mM of 2-DG; (▥) cells exposed to 10 mM 2-DG and 10 mM of lithium chloride simultaneously; (▨) cells pre-treated with 10 mM of lithium chloride for 24 h before 2-DG was added. Data are the mean ± SE of $n = 60$ EC, $^{*}P < 0.05$ vs. non-treated control, $^{\#}P < 0.05$ vs. 2-DG alone and the simultaneous addition of 2-DG and lithium.

Ca^{2+} discharge from the ER might prevent the secondary sustained influx of Ca^{2+}, which might be mediated by store-operated or Ca^{2+}-gated Ca^{2+} channels of the plasma membrane [17].

We used an established model of cultured monolayers of porcine aortic EC. These cells share the ubiquitous Ca^{2+} homoeostatic response to manoeuvres causing metabolic inhibition with coronary microvessel endothelial cells [12] and the intact aortic intima [23]. In addition, EC generally respond with a significant decrease in cellular ATP, not only via a combined glycolytic and mitochondrial inhibition, as previously reported [22], but also by solitary inhibition of glycolysis.

Ca^{2+} may be released from ER via two principle routes in EC involving the ryanodine and/or IP_3 receptors [21]. Our group has previously demonstrated that the contribution of ryanodine receptors can be excluded in this process [23]. Therefore, the initial Ca^{2+}-release shown in the present study is most likely mediated by IP_3-receptors, which may open in response to an increase in cytosolic IP_3 levels, as has been observed under ischemic conditions [26]. The IP_3 dependent Ca^{2+} release of the ER may also be sensitised by the local drop in ATP at the ER in close vicinity of IP_3-receptors ([9,16,25]; for a review, see [10]).

In a previous study, we demonstrated that the sustained $[Ca^{2+}]_i$ increase is based on Ca^{2+} influx via the plasma membrane [19]. The biphasic character of the Ca^{2+} signal, and in particular the transient decrease of $[Ca^{2+}]_i$ following the peak of the initial phase, was shown to be due to functionally active Ca^{2+}-ATPases, which accomplish Ca^{2+}-restoration into the ER or outward transport of Ca^{2+} across the plasma membrane. As shown by the biphasic increase in $[Ca^{2+}]_i$ induced by moderate metabolic inhibition, there is an active restoration of Ca^{2+} counteracting the cytosolic Ca^{2+} overload. It remains questionable, however, whether the capacity of Ca^{2+}-ATPases is sufficient to compensate for the initial influx of Ca^{2+} and, most importantly, whether this capacity is sufficient to prevent the accumulation of Ca^{2+} when metabolic inhibition persists and ATP production falls short. Thus, we aimed to prevent $[Ca^{2+}]_i$ overload and the associated detrimental consequences using a clinically established, safe psychiatric drug [5,18]. We have previously shown that Xestospongin C (XeC), an inhibitor of IP_3-dependent Ca^{2+} release channels [11], prevents $[Ca^{2+}]_i$ overload in response to mediators [20] or metabolic inhibition [23]. Recently, it has been confirmed that XeC prevents recruitable and spontaneous $[Ca^{2+}]_i$ increases in EC [28]. However, this drug is presumably not safe for use in humans. Lithium, in contrast, does not affect cell viability in doses of 1–30 mM [8] and may therefore be a better candidate to treat $[Ca^{2+}]_i$ overload.

In the present study, we have proven this concept using a standard manoeuvre to interfere with inositol phosphate metabolism in EC. The 24-h pre-treatment with lithium chloride abolished the $[Ca^{2+}]_i$ overload induced by the inhibition of glycolytic ATP production. This manoeuvre was sufficient to prevent both the initial transient and the sustained Ca^{2+} increase. In line with this concept, lithium did not affect the increase in $[Ca^{2+}]_i$ when added to 2-DG, indicating that acute lithium effects, e.g., those due to inhibition of Ca^{2+} influx channels, can be excluded.

Taken together, the data in the present study show that lithium protects EC against $[Ca^{2+}]_i$ overload, one of the most common stress signals generated in EC in response to a plethora of pathophysiological conditions, such as hypoxia, ischemia/reperfusion or inflammation. The fact that long-term rather than acute treatment with lithium confers the preservation of EC structure and function suggests the involvement of mechanisms similar to those described for neurons in vitro and in vivo (for a review, see [6]). However, it remains unclear whether the same or alternative pathways are responsible for this potentially ubiquitous cytoprotective effect of lithium.

Some limitations of our study should be mentioned. First, we used a culture model of porcine aortic EC because this cell type shows general endothelial characteristics. This model was chosen for a first exploration; a priori, it does not represent the specific characteristics of all different endothelial territories. Thus, caution is needed to translate our results to EC with organ-specific functions, and the results have to be confirmed, e.g., in human cerebral EC. Second, we focused on the measurement of $[Ca^{2+}]_i$ because this second massager controls a variety of endothelial functions and particularly permeability. IP_3, endothelial gap formation and functional permeability were not investigated in this pilot study and are planned as next steps to explore strategies for the protection of endothelial barrier function.

In conclusion, lithium prevents the initial as well as the secondary $[Ca^{2+}]_i$ increase after metabolic inhibition and thus counteracts unfavourable consequences. The protective effect is most likely accomplished by a blockade of the IP_3-sensitive Ca^{2+}-release mechanism of the ER in endothelial cells. Though further research is needed, our findings may potentially illustrate a new therapeutic strategy to prevent endothelial dysfunction.

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