Ischemic acidosis causes apoptosis in coronary endothelial cells through activation of caspase-12

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Abstract

Objective: Myocardial ischemia has been shown to induce apoptosis of endothelial cells (EC). However, the mechanism of this endothelial injury is still poorly understood. To analyse the signaling pathway of ischemia-induced EC apoptosis was the aim of the present study.

Methods: The primary culture of rat coronary EC was exposed to simulated ischemia (glucose-free anoxia at pH 6.4). Apoptosis was defined by staining of nuclei with Hoechst-33342 and TUNEL. Cytosolic Ca2+ and pH were measured with Fura-2 and BCECF, respectively.

Results: Apoptosis (29.2±1.7% of cells) induced by exposure to simulated ischemia for 2 h was accompanied by cytosolic Ca2+ overload (1090±52 nmol/l) and acidosis (pHi = 6.52±0.13). Simulated ischemia had no significant effect on caspase-8 cleavage, but induced cleavage of caspase-3 and caspase-12 and led to a slight release of cytochrome C. Prevention of cytosolic acidosis (anoxia at pHo 7.4) had no effect on cytochrome C release, but significantly reduced apoptosis, attenuated cytosolic Ca2+ overload, and prevented cleavage of caspase-12. A similar effect was achieved by inhibition of Ca2+ release channels in the endoplasmic reticulum with ryanodine and xestospongin C. Knock-down of caspase-12 with small interfering RNA suppressed caspase-3 activation and reduced apoptotic cell number by about 70%.

Conclusion: Acidosis, rather than anoxia, is an important trigger of apoptosis in EC under simulated ischemia. The main pathway of the simulated ischemia-induced apoptosis consists of the Ca2+ leak from the ER followed by activation of caspase-12 and caspase-3.

Keywords: Coronary endothelial cell; Apoptosis; Acidosis; Caspase-12

1. Introduction

Increasing evidence suggests that apoptosis of endothelial cells (EC) can be responsible for acute and chronic coronary diseases, e.g. through atherogenesis [1], thrombosis [2] and endothelial dysfunction [3]. Myocardial ischemia has been shown to be an important trigger of apoptosis [4,5]. Although several attempts have been done to characterize the mechanisms of EC apoptosis induced by ischemia, the precise apoptotic pathway is still unclear.

Generally, ischemia may lead to apoptosis through activation of extrinsic and intrinsic signaling pathways. The extrinsic pathways are associated with activation of death receptors (e.g. with TNF-alpha) followed by cleavage of procaspase-8. Within intrinsic pathways, ischemia-induced mitochondrial stress, leading to the release of cytochrome C and activation of caspase-9 and caspase-3, was demonstrated to be an important apoptotic pathway...
under various stress conditions including ischemia [5]. Additionally to mitochondria, endoplasmic reticulum (ER)-dependent apoptosis was intensively analysed in the past years in relation to cancer [6], neurodegenerative diseases [7] and cerebral ischemia [8]. Stress-induced accumulation of misfolded proteins in the ER results to ER-stress [9] followed by activation of ER-bound caspase-12 and apoptosis [10]. Involvement of the ER-pathway was recently demonstrated for apoptosis of human umbilical vein EC challenged with lipopolysaccharides [11]. Whether the ER-pathway may participate in apoptosis of coronary EC under ischemic conditions remains unclear.

In our previous study [12] we found that simulated ischemia leads to excessive accumulation of Ca\textsuperscript{2+} in the cytosol of EC mainly due to the leak of Ca\textsuperscript{2+} from the ER. Uncontrolled Ca\textsuperscript{2+}-leak from the ER has been shown to induce the ER-dependent apoptosis [13,14]. Therefore, we hypothesized that the ER-dependent pathway may contribute to apoptosis of coronary EC under ischemic insult. To prove this hypothesis and to analyse the pathway of ischemia-induced apoptosis a primary culture of microvascular coronary EC isolated from rat heart exposed to in vitro simulated ischemia (glucose-free anoxia, pH\textsubscript{o} 6.4) was applied as an experimental model. We found that simulated ischemia leads to Ca\textsuperscript{2+}-leak from the ER in acidosis-dependent manner followed by cleavage of ER-bound caspase-12 and apoptosis.

2. Methods

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, revised 1996).

2.1. Cell culture

Coronary EC were isolated from 250 to 300 g male Wistar rats and maintained in culture as previously described [15] in Eagle’s minimal essential medium 199 supplied with 10% fetal calf serum and 10% newborn calf serum. Before the experiments serum content in the culture medium was reduced from 20% to 5% for 18 h. Experiments were performed with monolayers reaching 90% confluence. The purity of the cell culture (>95% EC) was confirmed by immunochemical staining with antibodies against vWF and by uptake of Dil-ac-LDL as described previously [16].

2.2. In vitro simulated ischemia

To simulate ischemic conditions cells were treated with anoxia in combination with glucose deprivation and acidosis as described previously [12]. Dishes were incubated for 2 h at 37 °C in a gas-tight chamber under continuous flush with humidified gas mixture (95% N\textsubscript{2} + 5% CO\textsubscript{2}). Analysis of buffer pH after 2 h of simulated ischemia did not reveal any significant alteration.

2.3. Caspase-3 activity assay

Activity of caspase-3 in cell extracts was detected using a calorimetric caspase-3 cellular activity assay kit (Calbiochem) based on cleavage of the synthetic caspase substrate-1 linked to the chromophore p-nitroanilide (Ac-DEVD-pNA). Preparation of cell extracts and analysis of caspase-3 activity was performed according to the manufacturer’s protocol. The amount of hydrolyzed substrate was measured as an optical density at 405 nm. The activity of caspase-3 was expressed in arbitrary units defined as the maximal increase of optical density, derived by linear regression, per 0.5 × 10\textsuperscript{6} cells for 30 min.

2.4. Hoechst-33342 and propidium iodide staining

To distinguish between apoptotic and necrotic cells staining of nuclei with Hoechst-33342 and propidium iodide was applied as described previously [17] with modifications. Briefly, cells were trypsinized, washed with PBS and incubated for 10 min with 1 μg/ml Hoechst-33342 and 5 μg/ml propidium iodide. The stained nuclei were visualized with convert fluorescent microscope at a magnification of 700× using excitation light at 350 nm for Hoechst-33342 and 540 nm for propidium iodide.

For quantitative assay, a blind analysis of 200–300 nuclei from randomized 4–5 fields was applied. Cells were scored as apoptotic, when nuclei stained with Hoechst-33342 produced unequivocal bright blue fluorescence due to chromatin condensation/fragmentation (Fig. 1A). Propidium iodide stained nuclei with normal nuclear morphology, i.e. without signs of chromatin condensation, were scored as necrotic. Cells exhibiting both chromatin alteration and propidium iodide stained nuclei (i.e. “late-stage apoptotic cells”) were included in apoptotic population. The number of these cells did not exceed 5% of all cells.

2.5. TUNEL staining

TUNEL staining using the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostic GmbH, Mannheim) was performed according to the manufacturer’s instructions. Samples were analysed with a Leica TCS SP2 confocal microscope. Four culture dishes (each 200–300 cells) per group were used for the quantification of TUNEL-positive cells.

2.6. Western blot

Western blot analyses were performed as described previously [18]. Primary antibodies were: cytochrome C (Sigma), cytochrome oxidase IV (Molecular Probes), beta-actin (Chemicon International), caspase-3 (BD Biosciences), caspase-8 (Bio-Vision), caspase-12 (recognizing the residues 95-318, Sigma) and caspase-12 (recognizing the residues 100–116, Oncogene). Specific bands were visualized after
incubation with peroxidase-linked/HRP-labeled secondary antibodies by chemiluminescence using ECL+ kit (Amer-
sham Pharmacia). Equivalent sample loading was confirmed by stripping membranes with the Blot Restore Membrane Stripping buffer (Pierce) followed by treatment with antibodies against beta-actin.

2.7. Assay of cytochrome C release from mitochondria

Separation of mitochondrial and cytosolic fractions was performed as described previously [19]. Equal amounts of total proteins (50 μg for cytosolic fraction and 20 μg for mitochondrial fraction) were separated by 15% SDS-PAGE and then analysed by Western blot as described above. Analysis of beta-actin and cytochrome oxidase IV (Cox-IV) was used to confirm the equivalent loading of samples from cytosolic and mitochondrial fractions, subsequently. The purity of cytosolic fraction was confirmed by the absence of Cox-IV.

2.8. Procaspase-12 RNA Interference

The siRNA duplexes, corresponding to separate regions within the rat caspase-12 RNA sequence (Accession number NM_130422, Cat.Nr: M-080093-00) and non-targeting siRNA duplexes (Cat.Nr: D-001210-01) were purchased from Dharmacon Research (Lafayette, CO, USA). Cells were transfected according to the manufacturer’s instructions. Briefly, cells were seeded one day before transfection in MEM containing 10% FBS without antibiotics. Procaspase-12 siRNA or nRNA were mixed with oligofectamine (Invitrogen) in OptiMEM (Gibco BRL) for 15 min at room temperature and then added to culture medium with the final concentration of 50 nM of either oligonucleotide. Cells were
incubated at 37 °C for 48 h. Protein expression was determined by Western blot using specific antibody to procaspase-12, which revealed >90% reduction of procaspase-12.

2.9. Cytosolic Ca$^{2+}$ and pH analysis

Measurement of cytosolic-free Ca$^{2+}$ and H$^+$ concentration with fluorescent indicators Fura-2 and BCECF and calibration of Fura-2 and BCECF ratios were performed as previously described [12].

2.10. Statistical analysis

Data are given as mean±SEM. For each experimental protocol own randomized positive (no treatment) and negative (anoxia at pHo 6.4) control experiments were performed. The comparison of means between the groups was performed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Statistical significance was accepted when $p<0.05$.

3. Results

3.1. Ischemic acidosis is an important trigger of apoptosis in coronary EC

Exposure of EC to simulated ischemia for 2 h led to the increase of cells with chromatin condensation or fragmentation revealed by nuclei staining with Hoechst-33342 (Fig. 1A). This was accompanied by cleavage (Fig. 1B) and increased activity of caspase-3 (Fig. 2A). Compared to apoptotic cells, number of necrotic cells was only slightly increased (Fig. 2A).

Extracellular acidosis is a relevant stress factor during ischemic insult and can participate in cell death [18,20,21]. To test the role of acidosis in EC apoptosis, cells were incubated for 2 h in anoxic, glucose-free medium at pHo 7.4. Under this condition caspase-3 activity and the number of apoptotic cells were significantly reduced (Fig. 2A). Prevention of extracellular acidosis also slightly, but significantly reduced the number of necrotic cells. To confirm further the importance of low extracellular pH for apoptosis, EC were challenged with acidosis under normoxia, i.e. incubation of EC in normoxic, glucose-contained medium at pHo 6.4 for 2 h. Here, similar apoptosis and caspase-3 activity were defined as under simulated ischemia (Fig. 2A).

Additionally, the effect of acidosis was tested in serum-free normoxic cell culture medium. A significant acidosis-induced increase in caspase-3 activity (arbitrary units: 0.0171±0.0010 at pHo 6.4 vs. 0.0077±0.0014 at pHo 7.4, $n=3$, $p<0.01$) and the number of apoptotic cells (27.8 ±2.4% at pHo 6.4 vs. 13.7 ±1.3% at pHo 7.4, $n=3$, $p<0.01$) was observed. Therefore, acidosis, rather then anoxia and glucose

Fig. 2. (A) Caspase-3 activity and the number of apoptotic and necrotic EC under control condition or after 2 h of the following treatment: anoxia at pHo 6.4 (A6.4); anoxia at pHo 7.4 (A7.4); normoxia at pHo 6.4 (N6.4). Values are mean±SEM, $n=5$ to 9.⁎ — $p<0.05$ vs. A6.4. (B) Time courses of cytosolic pH and Ca$^{2+}$ concentration in EC under anoxia at pHo 6.4 (A6.4, – ● –) or pHo 7.4 (A7.4, – ▴ –), or under normoxia at pHo 6.4 (N6.4, – ○ –). Values are mean±SEM, $n=11$ to 16.⁎ — $p<0.05$ vs. A6.4.
deprivation, seems to be a key ischemic component triggering apoptosis in coronary EC under simulated ischemia.

3.2. Simulated ischemia leads to Ca\(^{2+}\) overload and cytosolic acidification

Disturbance of cytosolic Ca\(^{2+}\) and pH homeostasis is an essential event leading to apoptosis in stressed cells [13,20]. In the present study, exposure of EC to glucose-free anoxia at pH\(_{o}\) 6.4, i.e. simulated ischemia, led to a rapid acidification of the cytosol, reaching after 2 h the pH value of 6.52±0.13 (n=16) (Fig. 2B). Incubation of EC at pH\(_{o}\) 6.4 under normoxic condition also produced a pronounced cytosolic acidification (6.65±0.16, n=14, NS vs. anoxia at pH\(_{o}\) 6.4). In contrast, treatment of EC with anoxia at pH\(_{o}\) 7.4 produced only a small decline of the cytosolic pH.

Incubation of EC in anoxic or normoxic medium at pH\(_{o}\) 6.4 induced continuous rise of cytosolic Ca\(^{2+}\) concentration (Fig. 2B), leading after 2 h to cytosolic Ca\(^{2+}\) overload (1090±52 nmol/l (n=14) and 889±81 nmol/l (n=12), respectively). Prevention of cytosolic acidification during anoxia by increasing of the extracellular pH to 7.4 significantly suppressed Ca\(^{2+}\) overload. Therefore, simulated ischemia leads to Ca\(^{2+}\) overload in EC in a pH-dependent manner.

3.3. Ca\(^{2+}\)-leak from the ER triggers apoptosis in coronary EC

To test whether attenuation of the Ca\(^{2+}\) overload may protect EC against simulated ischemia-induced apoptosis, cells were treated during simulated ischemia with 3 \(\mu\)mol/l ryanodine and 3 \(\mu\)mol/l xestospongin C, the inhibitors of ryanodine- and IP\(_{3}\)-sensitive Ca\(^{2+}\) release channels in the ER, respectively. This approach was based on our previous data demonstrating that leak of Ca\(^{2+}\) from the ER, rather than extracellular Ca\(^{2+}\) influx, is an important route for Ca\(^{2+}\) accumulation in EC under simulated ischemia [12]. Treatment with ryanodine and xestospongin C significantly reduced ischemic Ca\(^{2+}\) accumulation (522±42 nmol/l, n=12, p<0.05 vs. anoxia at pH\(_{o}\) 6.4 without treatment, Fig. 3A). This Ca\(^{2+}\)-effect was accompanied by a marked reduction of caspase-3 activity and the apoptotic cell number (Fig. 3B).

3.4. Ca\(^{2+}\)-leak from the ER leads to activation of caspase-12

It has previously been shown that uncontrolled leak of Ca\(^{2+}\) from the ER can trigger the ER-stress followed by cleavage of ER-bound caspase-12 and apoptosis [13]. To find out whether the cleavage of caspase-12 is the link between acidosis-induced Ca\(^{2+}\)-leak from the ER and apoptosis, the analysis of the cleaved form of caspase-12 (32 kDa) was performed by Western blot. It was found that simulated ischemia markedly increased the cleaved form of caspase-12 (Fig. 4A). Prevention of ischemic cell acidification by anoxic incubation at pH\(_{o}\) 7.4 or inhibition of Ca\(^{2+}\)-leak from the ER during simulated ischemia with ryanodine and xestospongin C abolished cleavage of caspase-12.

To demonstrate the coherence between caspase-12 cleavage and the rate of apoptosis under different experimental conditions, a second method of the apoptosis detection was applied, i.e. TUNEL staining of nuclei (Fig. 4B).
This apoptosis analysis method demonstrated similar alteration of apoptotic EC number as defined by Hoechst-33342 staining (see above). A clear association between caspase-12 cleavage and the rate of EC apoptosis can be observed.

### 3.5. Antisense knock-down of caspase-12 protects against simulated ischemia-induced apoptosis

To prove the causal role of caspase-12 in ischemia-induced apoptosis of EC, the down-regulation of procaspase-12 was performed by pre-treatment with siRNA. This treatment reduced procaspase-12 expression by about 90% (Fig. 5A). Analysis of apoptotic cell death demonstrated that siRNA treatment significantly suppressed ischemia-induced caspase-3 activation and reduced the apoptotic cells number (10.8±2.6%), whereas treatment with non-targeting siRNA had no significant effects (Fig. 5B). Under both conditions the necrotic cell death was unchanged. Similar reduction of apoptotic cell death was produced by treatment under simulated ischemia with pan-caspase inhibitor zVAD-fmk, demonstrating the predominance of caspase-dependent apoptosis.

### 3.6. Mitochondria- and death receptor-mediated pathways are not involved in acidosis-dependent apoptosis

To investigate the role of other apoptotic pathways, mitochondrial release of cytochrome C and cleavage of caspase-8 were analysed. We found that simulated ischemia led to elevation of cytosolic cytochrome C. This release of cytochrome C was, however, slight, because no alteration of mitochondrial cytochrome C content could be detected under simulated ischemia (Fig. 6). Additionally, the cytochrome C release was similar under anoxic incubation of EC at pH 6.4 or 7.4 indicating independence of simulated ischemia-induced cytochrome C release of acidosis.

To prove the contribution of the death receptor-mediated pathway in simulated ischemia-induced apoptosis of EC, cleavage of caspase-8 was analysed. No cleavage of...
caspase-8 could be detected after exposure of EC to simulated ischemia (data not shown).

4. Discussion

The aim of the present study was to analyse the signaling pathways of simulated ischemia-induced apoptosis in coronary EC. The main findings are the following: (i) ischemic acidosis, rather than oxygen and glucose deprivation, is an important trigger of apoptosis in coronary EC. (ii) The main signaling pathway of the simulated ischemia-induced apoptosis includes pH-dependent Ca2+-leak from the ER and cleavage of ER-bound caspase-12 followed by cleavage of caspase-3.

To simulate ischemia, a combination of anoxia with extracellular acidosis (pHo 6.4) was performed in the present study. This extracellular acidification seems to be relevant to in vivo myocardial ischemia, where extracellular pH can decrease to as low as 6.0 [22]. Previous studies demonstrated the link between acidosis and apoptosis in various cell types, e.g. cardiomyocytes [20], astrocytes [18], neutrophils [23] and tumor cells [24]. Whether acidosis may also be responsible for apoptotic death of EC under ischemic insult was unclear. In the present study two lines of evidence clearly demonstrated the importance of cytosolic acidosis for apoptosis of EC. First, prevention of cytosolic acidification during anoxia by the increase of pHo to 7.4 significantly attenuated simulated ischemia-induced EC apoptosis. Second, simulation of ischemic extracellular acidosis under normoxic conditions led to a similar cytosolic acidification and apoptosis of EC as simulated ischemia itself. Thus acidosis, rather than oxygen and glucose deprivation, seems to be a trigger of EC apoptosis under simulated ischemia. In agreement with this finding, previous study on cardiac myocytes demonstrated that hypoxia leads to apoptosis only in combination with acidosis [20]. In contrast to the pro-apoptotic effect of the severe extracellular acidification (pHo 6.4) observed in our study, mild acidosis (pHo 7.0) has been shown to produce an opposite, antiapoptotic effect [25]. Obviously, this mild form of extracellular acidification is not sufficient to induce apoptosis. In fact, there seems to be a correlation between the observed extracellular pH value and the apoptosis rate. As demonstrated in human atrial tissue and neonatal rat cardiac myocytes extracellular pH values at about 7.0 did not induce apoptosis, whereas extracellular pH values <6.5 go along with a significant increase in apoptosis [20,21].

We found that the acidification in EC was accompanied by accumulation of Ca2+ in the cytosol regardless of oxygen and glucose deprivation, suggesting that cytosolic Ca2+ overload could be an important link between acidosis and apoptosis in EC. Previously we have demonstrated that the leak of Ca2+ from the ER, rather than the extracellular Ca2+ influx, is a main route for accumulation of Ca2+ in the cytosol of coronary EC under similar simulated ischemia [12]. In the present study treatment with ryanodine and xestospongin-C was applied in order to reduce the Ca2+-leak from ryanodine- and IP3-sensitive Ca2+-stores. The expression of ryanodine receptors in nonexcitable cell types, like EC, is debatable. Nevertheless, the importance of ryanodine-sensitive Ca2+-store for simulated ischemia-induced cytosolic Ca2+ overload in coronary EC was verified in our previous study [12]. Additionally, in situ expression of ryanodine receptor [26] and functional role of ryanodine-sensitive Ca2+ channels in EC [27] was previously also demonstrated. In line with previous data [12], inhibition of Ca2+ release channels in the ER with ryanodine and xestospongin C in the present study significantly reduced simulated ischemia-induced Ca2+ overload and suppressed apoptosis. This finding indicates that the pH-dependent leak of Ca2+ from the ER plays an important role for apoptosis of EC under ischemic conditions.

Several studies have reported that uncontrolled depletion of Ca2+ in the ER may lead to ER-stress followed by cleavage of ER-bound caspase-12 [13,14]. In agreement with these data, cleavage of caspase-12 was found in the present study in acidosis-dependent manner, demonstrating the link between acidosis and caspase-12. Importantly, the cleavage of caspase-12 was also abolished by inhibition of Ca2+ release channels in the ER. Therefore, acidosis-induced Ca2+-leak from the ER is an essential event leading to activation of caspase-12.
To show the causal role of caspase-12 cleavage for EC apoptosis, specific siRNA-mediated knock-down of procaspase-12 gene was performed, which produced about 90% suppression of procaspase-12 expression and completely prevented the appearance of cleaved form of caspase-12 under simulated ischemia (Fig. 5A). Under this condition, elevation of caspase-3 activity and apoptotic cell number by simulated ischemia was reduced by about 70%. This antiapoptotic effect of siRNA treatment seems to be specifically due to prevention of procaspase-12 expression, since control treatment with non-targeting siRNA had no effect. Thus, cleavage of caspase-12 is a key event in simulated ischemia-induced apoptosis of coronary EC.

The remaining caspase-3 activity and apoptosis rate (about 30%) induced by simulated ischemia could be due to activation of other, caspase-12-independent pathways, e.g. (i) receptor-mediated activation of caspase-8, (ii) mitochondrial pathways or (iii) caspase-independent pathways. Receptor-mediated pathway seems to be not important in our model, since the cleaved form of caspase-8 could not be detected. This finding is in agreement with a previous study on the whole heart, demonstrating that in coronary EC caspase-8 is not activated under myocardial ischemia/reperfusion [28]. In contrast, mitochondrial pathway seems, at least partly, to be involved in simulated ischemia-induced apoptosis. Indeed, the slight but significant release of cytochrome C was detected in EC during ischemic exposure. However, it was not responsible for acidosis-dependent apoptosis, since the cytochrome C release was pH₃⁻ independent. These data are in discrepancy with previously demonstrated involvement of mitochondria in ER-Ca²⁺ release-induced apoptosis in EC [29]. The reason for the discrepancy may be related to the difference in experimental models, e.g. O₂⁻ treatment [29] vs. simulated ischemia in our study. Particularly, the acidic milieu in our study may attenuate Ca²⁺-dependent mitochondrial injury under simulated ischemia by reduction of mitochondrial Ca²⁺-uptake [30] or by inhibition of the opening of permeability transition pores in the mitochondria [31]. Finally, caspase-independent apoptosis may participate in our study. Treatment with pan-caspase inhibitor zVAD-fmk markedly, but not completely, suppressed the rise of apoptotic cell number (Fig. 5B). Previously, activation of deoxyribonuclease II under acidic conditions (pH 6.5) followed by DNA degradation and apoptosis was demonstrated [32]. Thus, involvement of this acidosis-dependent mechanism of apoptosis in our model can not be excluded. However the mitochondrial or caspase-independent mechanisms might participate in apoptosis in this study, they play only a minor role compared with the caspase-12-dependent pathway.

The down-stream pathway of caspase-12-dependent apoptosis has been investigated previously and may include either a direct translocation of caspase-12 to the nucleus [33] or induction of proteolytic cascade through activation of caspase-9 followed by activation of caspase-3 [10]. Although the detailed analysis of the down-stream pathway was beyond the scope of the present study, activation of caspase-3 may be suggested as a link between caspase-12 activation and apoptosis of coronary EC in the present study. First, the increase of caspase-3 cleavage and activity was demonstrated under simulated ischemia, which correlated with the rise of apoptotic cell number and caspase-12 cleavage under different experimental conditions. Second, down-regulation of procaspase-12 expression was associated with a significant suppression of caspase-3 activity under simulated ischemia. Altogether, these data confirmed the previously described link between caspase-12 and caspase-3 [10] also for ischemic EC.

Although the present and other studies performed on rodents demonstrate the involvement of caspase-12 in apoptosis, the extrapolation of these animal data to humans should be done with precautions. Human caspase-12 gene has been shown to be transcribed into mRNA. However mature caspase-12 protein is not produced [34]. Thus, human caspase-12 does not seem to function in ER-dependent apoptosis. Nevertheless, in agreement with the present study, the involvement of ER-dependent pathway in apoptosis for human cells was demonstrated previously [11,35]. The recent study of Hitomi et al. also suggests that human caspase-4, which has 48% homology at the amino acid level with rodent caspase-12 and belongs to the same interleukin-Ibeta converting enzyme subfamily, corresponds to rodent caspase-12 to initiate cell death signaling pathway induced by ER-stress [35]. Thus, though different caspases are involved in processing of ER-dependent apoptosis in rodents and humans, the importance of ER-pathway in ischemia-induced EC death could still be supposed for humans. Interestingly, the recent study of Thatte et al. [21] demonstrated a close correlation between acidosis and induction of apoptosis in cardiac samples obtained from patients undergoing cardiac surgery. The mechanisms of this acidosis-induced apoptosis in human heart are still unclear. According to the finding of the present study, the involvement of the ER-pathway may be suggested.

In conclusion, this study for the first time demonstrates the importance of acidosis for apoptosis of coronary EC under ischemic conditions and uncovers the mechanism of this simulated ischemia-induced apoptosis, i.e. Ca²⁺-leak from the ER and cleavage of ER-bound caspase-12. Therefore, the ER-dependent pathway plays an essential role in ischemia-induced apoptosis of coronary EC. Further investigation of this apoptotic pathway in coronary EC may lead to the discovery of new therapeutic approaches for the treatment of diseases accompanied with acidosis, e.g. coronary artery disease and inflammation.

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References


