

Acidic pre-conditioning suppresses apoptosis and increases expression of Bcl-xL in coronary endothelial cells under simulated ischaemia

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Received: May 4, 2007; Accepted: November 12, 2007

Abstract

Ischaemic pre-conditioning has a powerful protective potential against ischaemia-induced cell death, and acidosis is an important feature of ischaemia and can lead to apoptosis. Here we tested whether pre-conditioning with acidosis, that is, acidic pre-conditioning (APC), may protect coronary endothelial cells (EC) against apoptosis induced by simulated ischaemia. For pre-conditioning, EC were exposed for 40 min. to acidosis (pH 6.4) followed by a 14-hrs recovery period (pH 7.4) and finally treated for 2 hrs with simulated ischaemia (glucose-free anoxia at pH 6.4). Cells undergoing apoptosis were visualized by chromatin staining or by determination of caspase-3 activity. Simulated ischaemia in untreated EC increased caspase-3 activity and the number of apoptotic cell ($31.3 \pm 1.3\%$ versus $3.9 \pm 0.6\%$ in control). APC significantly reduced the rate of apoptosis ($14.2 \pm 1.3\%$) and caspase-3 activity. Western blot analysis exploring the underlying mechanism leading to this protection revealed suppression of the endoplasmic reticulum- (reduced cleavage of caspase-12) and mitochondria-mediated (reduced cytochrome C release) pathways of apoptosis. These effects were associated with an over-expression of the anti-apoptotic protein Bcl-xL 14 hrs after APC, whereas no effect on the expression of Bcl-2, Bax, Bak, procaspase-12, reticulum-localized chaperones (GRP78, calreticulin), HSP70, HSP32 and HSP27 could be detected. Knock-down of Bcl-xL by siRNA-treatment prevented the protective effect of APC. In conclusion, short acidic pre-treatment can protect EC against ischaemic apoptosis. The mechanism of this protection consists of suppression of the endoplasmic reticulum- and mitochondria-mediated pathways. Over-expression of the anti-apoptotic protein Bcl-xL is responsible for the increased resistance to apoptosis during ischaemic insult.

Keywords: apoptosis • endothelial cells • ischaemia • acidosis • pre-conditioning • Bcl-xL • caspase-12 • cytochrome C

Introduction

Myocardial ischaemia/reperfusion leads to apoptosis of coronary endothelial cells (EC) [1], which may contribute to the pathogenesis of endothelial dysfunction [2], atherosclerosis [3] and thrombosis [4]. Among several protection strategies directed against ischaemic injury, ischaemic pre-conditioning, that is, adaptation to ischaemia through one or more short preceding ischaemic episodes, was intensively investigated during the last years and has been demonstrated to have a powerful protective potential against ischaemic cell death [5]. Since pre-treatment with ischaemia is clinically not applicable, many attempts have been

undertaken to achieve the protective effect of ischaemic pre-conditioning either pharmacologically [6] or by pre-treatment with different components of ischaemia, for example, hypoxia [7], glucose deprivation [8] or acidosis [9].

In our previous study [10] acidosis was found to be an important stress factor triggering apoptosis in coronary EC under ischaemic conditions. According to the pre-conditioning paradigm, 'almost any stress factor that is potentially harmful for cells can elicit a pre-conditioned state, *i.e.* increased resistance to the damaging stress, when applied in small quantities' [5]. Therefore, we hypothesized that a short acidic pre-treatment, that is, acidic pre-conditioning (APC), may protect EC against ischaemic apoptosis. To our knowledge, only one study applying an isolated heart model demonstrated the protective effect of APC against ischaemic injury with respect to myocardial necrosis and heart function [9]. However, whether APC can affect the apoptotic cell death was unknown. Therefore, the goal of the present study was

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to analyse whether APC may be protective against ischaemia-induced apoptosis. For this purpose, coronary EC were exposed to simulated ischaemia (glucose-free anoxia, pH 6.4) with or without pre-treatment with acidosis. We found that APC significantly reduced the apoptotic rate of ischaemic EC owing to suppression of endoplasmic reticulum- and mitochondria-mediated pathways of apoptosis and that this phenomenon was associated with an increased expression of the anti-apoptotic protein Bcl-xL.

Methods

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).

Cell culture

Coronary EC were isolated from 250- to 300-g male Wistar rats and maintained in Eagle's minimal essential medium 199 supplemented with 10% foetal calf serum and 10% newborn calf serum as previously described [11]. The purity of the cell culture (>95% EC) was confirmed by immunohistochemical staining with antibodies against vWF and by uptake of Dil-ac-LDL as previously described [12]. Experiments were performed with monolayers reaching 80–90% confluence, and 18 hrs prior to experiments serum content in the culture medium was reduced from 20% to 5%.

In vitro simulated ischaemia

To simulate ischaemic conditions, cells were treated with anoxia in combination with glucose-deprivation and acidosis as described previously [10]. Dishes were incubated for 2 hrs at 37°C in a gas-tight chamber under continuous flush with a humidified gas mixture (95% N₂ + 5% CO₂). Analysis of the buffer pH after 2 hrs of simulated ischaemia did not reveal any significant alteration.

Acidic pre-conditioning

Before simulated ischaemia, cells were exposed to acidosis in cell culture medium (pH 6.4) for 20–50 min. followed by a recovery period for 6–24 hrs in cell culture medium at pH 7.4. In control group, similar treatment, that is, changes of medium, was performed at pH 7.4.

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 the 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 0.5×10^6 cells within 30 min.

Hoechst-33342 and propidium iodide staining

To distinguish between apoptotic and necrotic cells, a staining of nuclei with Hoechst-33342 and propidium iodide was applied as described previously [13] 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. Stained nuclei were visualized with a converted fluorescence microscope at a magnification of 700x 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 as a result of chromatin condensation [10]. Propidium iodide-stained nuclei with normal nuclear morphology, that is, 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.

Western blot

Western blot analysis was performed as described previously [14]. Primary antibodies were cytochrome C (Sigma-Aldrich, Saint Louis, MO, USA); cytochrome oxidase IV (Molecular Probes, Eugene, OR, USA); Bax, Bak, Bcl-xL and Bcl-2 (Cell Signaling, Danvers, MA, USA); actin (Chemicon International, Temecula, CA, USA); HSP27 (Cell Signaling); HSP32 (Stressgen Bioreagents, BC, Canada); HSP70 (R&D Systems, Wiesbaden-Nordenstadt, Germany); calreticulin (BD Biosciences, San Diego, CA, USA); caspase-8 (Bio-Vision Research, Mountain View, CA, USA); GRP78 and caspase-12 (Calbiochem-Oncogene, Darmstadt, Germany). Specific bands were visualized after incubation with peroxidase-linked/HRP-labelled secondary antibodies by chemiluminescence using ECL+ kit (Amersham Pharmacia, Freiburg, Germany). For multiple analyses of different proteins, Western blot was performed from the same cell extract. Equivalent sample loading was confirmed by stripping membranes with the Blot Restore Membrane Stripping buffer (Pierce Biotechnology, Rockford, IL, USA) followed by treatment with antibodies against actin. Since the loading control was similar in all membranes, only one was shown in representative figures.

Analysis of cytosolic cytochrome C

Preparation of mitochondria-free cytosolic fractions was performed as described previously [15]. Equal amounts of total proteins (50 µg) were separated by 15% SDS-PAGE and then analysed by Western blot as described earlier. The purity of cytosolic fraction was confirmed by the absence of cytochrome oxidase IV.

siRNA transfection

Knock-down of Bcl-xL was achieved by treatment of EC with small interfering RNA duplexes (siRNA), corresponding to separate regions within the

rat Bcl-xL RNA sequence (Accession number NM_001033671, Cat. Nr: L-080091-01, Dharmacon Research, Lafayette, CO, USA). As a control, non-targeting siRNA duplexes (Cat. Nr: D-001810-01) were applied. Cells were transfected according to manufacturer's instructions. Briefly, cells were seeded 1 day before transfection in MEM containing 10% foetal bovine serum without antibiotics. Bcl-xL siRNA or non-targeting siRNA were mixed with oligofectamine (Invitrogen GmbH, Karlsruhe, Germany) in OptiMEM (Gibco BRL Invitrogen GmbH, Karlsruhe, Germany) for 15 min. at room temperature and then added to the culture medium at a final concentration of 60 nM. Cells were incubated at 37°C for 72 hrs. Protein expression was determined by Western blot using a specific antibody to Bcl-xL, which revealed $\geq 90\%$ reduction of Bcl-xL after 72 hrs.

Statistical analysis

Data are given as mean \pm S.E.M. The comparison of means between the groups was performed by ANOVA followed by Bonferroni post-hoc test. Statistical significance was accepted when P was < 0.05 .

Results

Effect of acidic pre-conditioning on the rate of apoptosis

Under control conditions, that is, incubation of endothelial cells (EC) in cell culture medium supplied with 5% serum for 16 hrs, only a few apoptotic cells ($3.9 \pm 0.6\%$, $n = 9$) and no significant caspase-3 activity was found. Exposure to simulated ischaemia for 2 hrs led to a significant increase in the number of apoptotic cells ($31.3 \pm 1.3\%$, $n = 14$) and caspase-3 activity (Fig. 1A). Pre-treatment of EC with acidosis in cell culture medium (pH 6.4) for 20–50 min. followed by a 14-hr recovery period at pH 7.4 induced a time-dependent reduction of ischaemia-induced apoptosis. The maximal protection against apoptosis was found after 40 min. acidic pre-treatment. Prolongation of the treatment up to 50 min. partially reversed the protective effect of APC. To optimize further the post-treatment period, that is, recovery period, cells were incubated for 6, 14 or 24 hrs after acidic treatment. Analysis of caspase-3 activity and the number of apoptotic cells revealed that 14 hrs of post-incubation led to a significantly better protection against apoptosis compared to 6 and 24 hrs (Fig. 1B). Therefore, 40 min. of acidic treatment followed by a 14-hr recovery period was found to be the optimal protocol for protection of EC against simulated ischaemia-induced apoptosis. A parallel determination of the necrotic cell number revealed no significant alteration of this parameter following simulated ischaemia without ($5.5 \pm 0.5\%$, $n = 14$) or with APC ($4.5 \pm 0.6\%$, $n = 10$) compared to controls ($5.9 \pm 0.6\%$, $n = 9$).

As a control, we further tested whether the short treatment with acidosis for 40 min. itself may lead to apoptosis of EC. This may eliminate during the 14-hr recovery period the cells susceptible to apoptosis and influence, therefore, the effect of simulated ischaemia on the apoptosis rate independently of increased resistance to ischaemic stress. The analysis of caspase-3 activity and

the number of apoptotic and necrotic cells demonstrated no significant alteration of these parameters during the 14-hrs recovery period compared to controls (Fig. 1C).

Acidic pre-conditioning suppresses caspase-12 cleavage and the release of cytochrome C

To find out which apoptotic pathway is predominantly suppressed by APC, first, the cleavage of endoplasmic reticulum (ER)-bound caspase-12 was investigated. This approach is based on our previous data demonstrating that caspase-12 cleavage is the major intrinsic pathway of simulated ischaemia-induced apoptosis in coronary EC [10]. In line with this report, a significant cleavage of caspase-12 was detected after simulated ischaemia in the present study. APC markedly reduced the cleavage of caspase-12, thus demonstrating the suppression of the ER-mediated pathway of apoptosis (Fig. 2).

In addition, we also investigated the participation of two other signalling pathways previously described for ischaemia-induced apoptosis, that is, the death receptor- and mitochondria-mediated pathways. For the potential contribution of the death receptor-mediated pathway, cleavage of caspase-8 was analysed. No cleavage of this caspase could be detected after exposure of EC to simulated ischaemia under any experimental condition (data not shown). To further analyse a possible role of the mitochondrial pathway, a release of cytochrome C from mitochondria was investigated. We found that simulated ischaemia led to the elevation of cytosolic cytochrome C, which was significantly attenuated by APC (Fig. 2). Thus, APC suppressed both, ER- and mitochondria-mediated pathways of apoptosis.

Acidic pre-conditioning did not affect expression of ER chaperones and procaspase-12

Cleavage of ER-bound caspase-12 is a result of ER stress, which may be suppressed by over-expression of ER-localized chaperones. Therefore, to test whether APC suppressed caspase-12 cleavage owing to an increased content of ER chaperones, Western blot analysis of GRP78 and calreticulin expression was performed 14 hrs after pre-conditioning, before simulated ischaemia. No changes in the steady-state expression of these chaperone proteins were found (Fig. 3). In addition, the analysis of procaspase-12 expression, which might influence the amount of cleaved caspase-12 during simulated ischaemia, also did not reveal any significant alteration in our study (Fig. 3).

Acidic pre-conditioning did not affect expression of heat shock proteins

Aside from alterations in GRP78 and calreticulin expression, enhanced cellular levels of heat shock proteins (HSPs) have been reported to

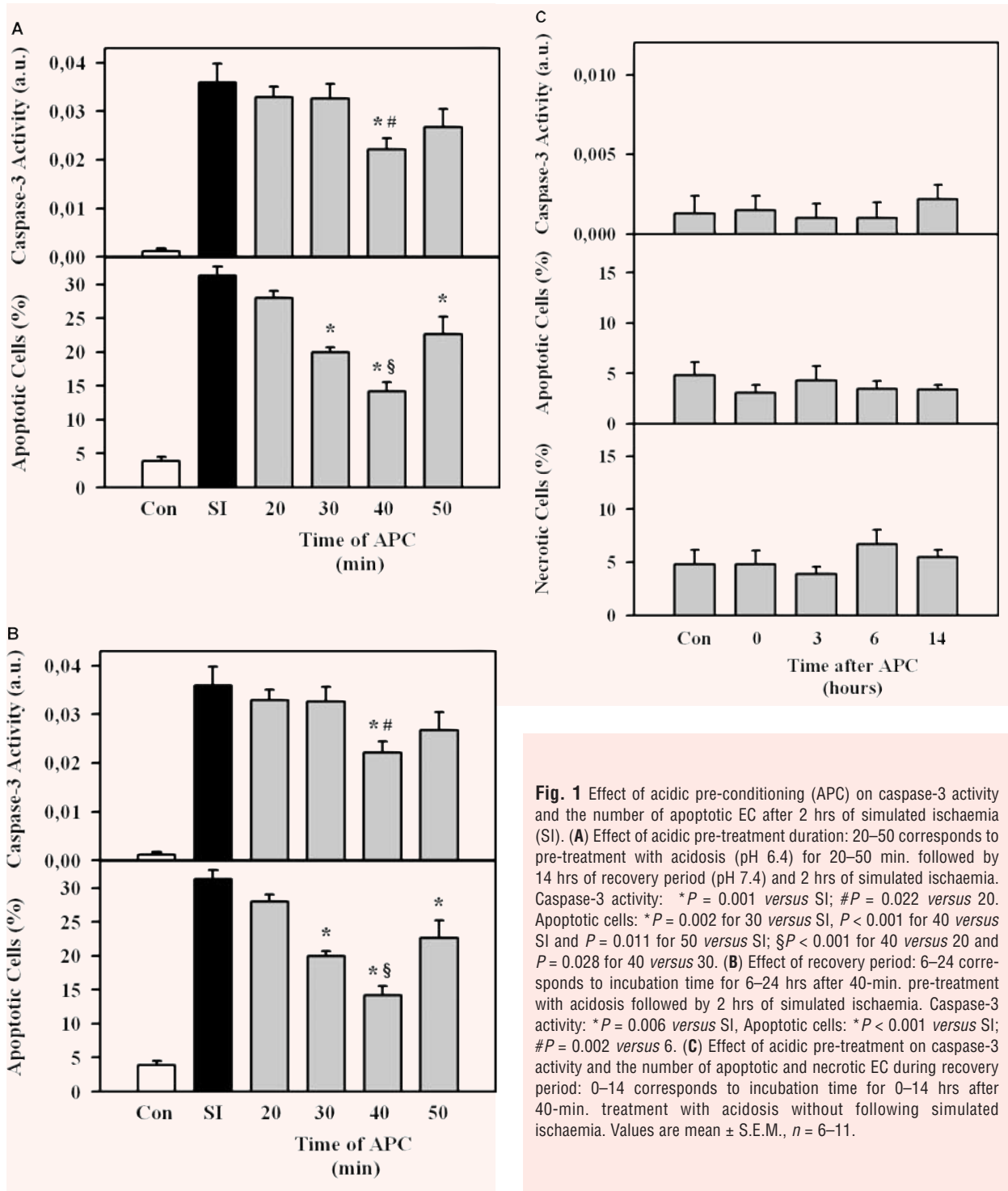


Fig. 1 Effect of acidic pre-conditioning (APC) on caspase-3 activity and the number of apoptotic EC after 2 hrs of simulated ischaemia (SI). **(A)** Effect of acidic pre-treatment duration: 20–50 min. followed by 14 hrs of recovery period (pH 7.4) and 2 hrs of simulated ischaemia. Caspase-3 activity: **P* = 0.001 versus SI; #*P* = 0.022 versus 20. Apoptotic cells: **P* = 0.002 for 30 versus SI, *P* < 0.001 for 40 versus SI and *P* = 0.011 for 50 versus SI; §*P* < 0.001 for 40 versus 20 and *P* = 0.028 for 40 versus 30. **(B)** Effect of recovery period: 6–24 hrs after 40-min. pre-treatment with acidosis followed by 2 hrs of simulated ischaemia. Caspase-3 activity: **P* = 0.006 versus SI, Apoptotic cells: **P* < 0.001 versus SI; #*P* = 0.002 versus 6. **(C)** Effect of acidic pre-treatment on caspase-3 activity and the number of apoptotic and necrotic EC during recovery period: 0–14 hrs after 40-min. treatment with acidosis without following simulated ischaemia. Values are mean ± S.E.M., *n* = 6–11.

be induced by ischaemic pre-conditioning and can be protective against apoptosis [16]. Therefore, we tested whether APC may also increase steady-state expression of HSPs. Western blot analysis for

HSP70, HSP32 and HSP27 expression was performed 14 hrs after APC, before simulated ischaemia. Surprisingly, no marked changes in the expression of these proteins could be detected (Fig. 4).

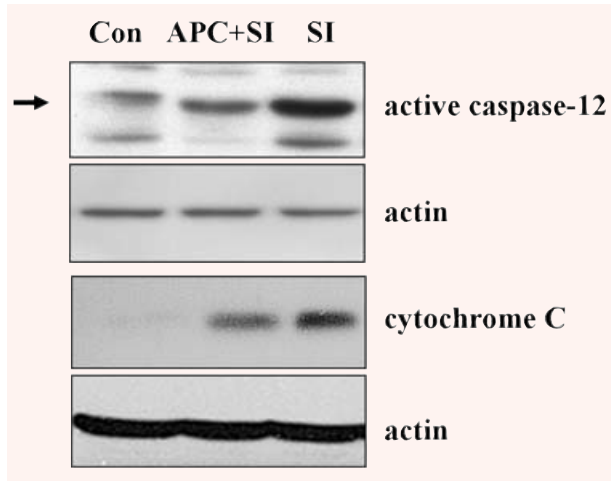


Fig. 2 Western blot analysis of cytosolic cytochrome C (A) and cleaved caspase-12 (B) prepared from extracts of control EC (Con) or EC exposed to simulated ischaemia (SI) without or after 40-min. acidic pre-treatment followed by a 14-hrs recovery period (APC + SI). Data are representative of four independent experiments with similar results.

Acidic pre-conditioning augmented expression of Bcl-xL

Finally, we analysed the expression of Bcl-2 family proteins, known to be important regulators of apoptosis [17]. To test whether the observed anti-apoptotic effect of APC is associated with an alteration in the expression of these proteins, Western blot analysis of Bax, Bak, Bcl-2 and Bcl-xL proteins was performed. No significant changes were found in the expression of Bax, Bak and Bcl-2. In contrast, expression of the anti-apoptotic protein Bcl-xL was significantly increased 14 hrs after APC (Fig. 5).

Bcl-xL knock-down abolished the protective effect of acidic pre-conditioning

To further prove the idea whether over-expression of Bcl-xL might be a key mechanism in the APC-induced protection against ischaemic apoptosis, knock-down of Bcl-xL was performed before simulated ischaemia by treatment of EC with Bcl-xL-specific siRNA. After 72-hrs treatment, the reduction of Bcl-xL by >90% was found (Fig. 6A). Subsequent analysis of apoptosis revealed that the Bcl-xL knock-down completely abolished the protective effect of APC (Fig. 6B). No significant difference in the number of necrotic cells was found between the groups.

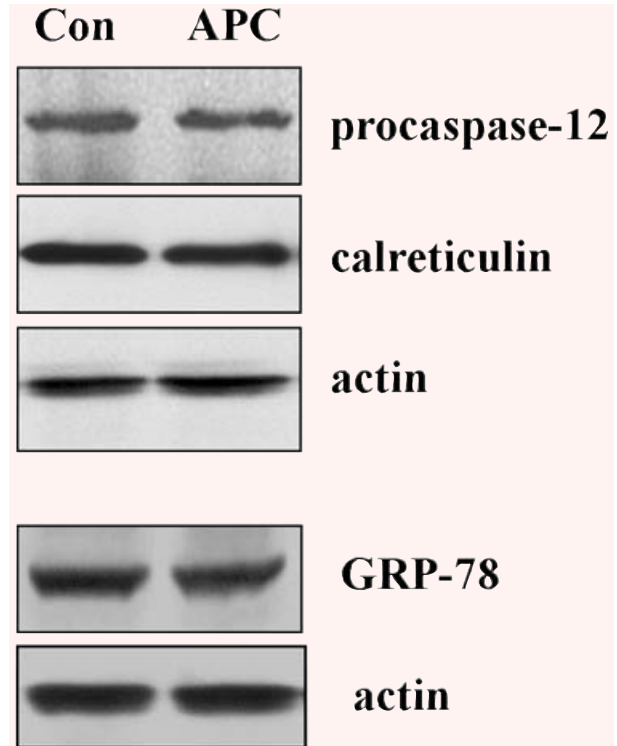


Fig. 3 Western blot analysis of procaspase-12, GRP78 and calreticulin prepared from extracts of control EC (Con) or EC after 40-min. acidic pre-treatment followed by a 14-hrs recovery period (APC). Data are representative of three to four independent experiments with similar results.

Discussion

The aim of the present study was to find out (i) whether acidic pre-conditioning (APC) can protect coronary EC against apoptosis induced by simulated ischaemia and (ii) which apoptotic pathways might be involved.

The main findings are the following: (i) APC leads to a significant reduction of ischaemia-induced apoptosis. (ii) The mechanism responsible for the anti-apoptotic action of APC involves the inhibition of ER- and mitochondria-mediated pathways. (iii) The protective effect of APC is associated with an over-expression of the anti-apoptotic protein Bcl-xL, which seems to play a key role in the APC-induced protection.

To simulate ischaemia, a combination of glucose-free anoxia with extra-cellular acidosis (pH 6.4) was applied in the present study. Such an extent of extra-cellular acidification is comparable to an *in vivo* myocardial ischaemia, where extra-cellular pH can drop to as low as 6.0 [18]. Our model of simulated ischaemia was characterized in previous studies, which suggested the importance of the acidification as an initial trigger for the cleavage of the ER-bound caspase-12 [10, 19] followed by an activation of

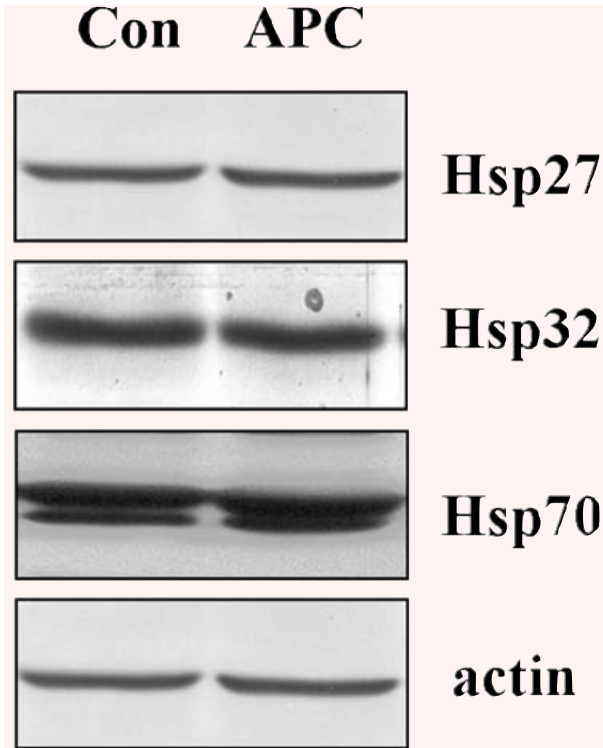


Fig. 4 Western blot analysis of HSP27, HSP32 and HSP70 prepared from extracts of control EC (Con) or EC after 40-min. acidic pre-treatment followed by a 14-hrs recovery period (APC). Data are representative of four independent experiments with similar results.

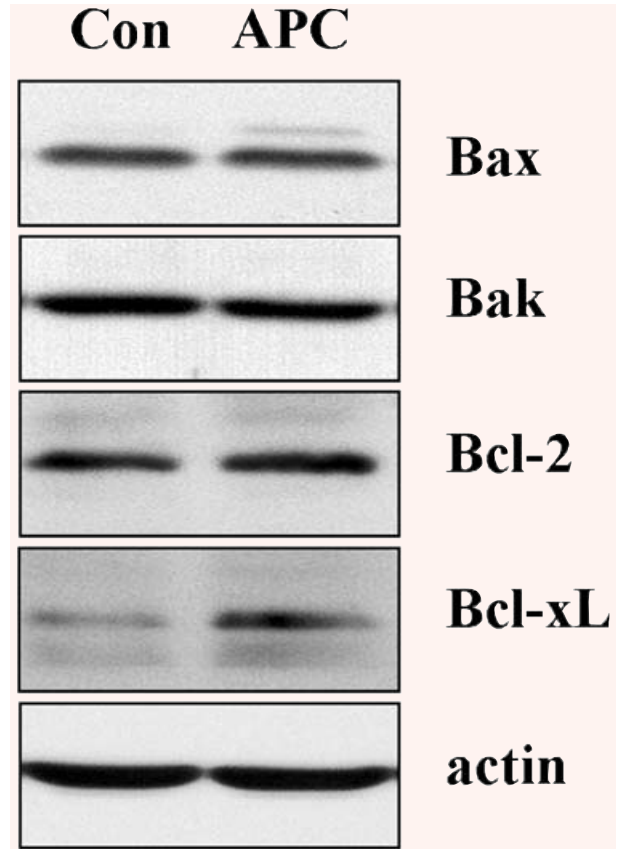


Fig. 5 Western blot analysis of Bax, Bak, Bcl-2 and Bcl-xL prepared from extracts of control EC (Con) or EC after 40-min. acidic pre-treatment followed by a 14-hrs recovery period (APC). Data are representative of three to four independent experiments with similar results.

caspase-3 and subsequent apoptosis. Several reports from other groups confirmed a pro-apoptotic potential of acidosis [14, 20]. Thus, cellular acidosis seems to be an important environmental stress factor leading to apoptosis.

In the present study, we proved the hypothesis that a short, harmless pre-treatment with acidosis may lead to an adaptation against the pro-apoptotic effect of simulated ischaemia. For this purpose EC were shortly exposed to a similar degree of acidosis (pH 6.4) that was used in our model of simulated ischaemia. Applying various protocols, we found that 40 min. of acidic exposure followed by a 14-hrs recovery period resulted in the best protective effect against ischaemic apoptosis.

A recent study by Thatte and colleagues [21] showed that 30-min. acidic exposure (pH 6.5) of human atrial tissue leads to a slight, but significant increase of apoptosis. Therefore, a similar acidic treatment (40 min., pH 6.4) during APC in our study may lead to a low level of apoptosis. This effect of APC may selectively eliminate cells susceptible to apoptosis and, thus, may be a cause for a reduced apoptotic rate during simulated ischaemia. However, during the 14-hr recovery period no increase of caspase-3 activity and no rise of apoptotic and necrotic cells numbers were detected.

Thus, 40-min. acidic treatment seems to be harmless for EC and does not result in an alteration of cell population with respect to vulnerability to apoptosis, but rather leads to an increased resistance of EC to ischaemia.

To discover the underlying molecular mechanisms responsible for this adaptation to ischaemic stress, the main known pathways of apoptosis were analysed. For this aim, cleavage of caspase-8, caspase-12 and release of cytochrome C were studied. Involvement of the death receptor pathway in our model is unlikely to be responsible for the APC effect, since no caspase-8 cleavage could be detected under any experimental condition. Cleavage of the ER-bound caspase-12 was previously reported to be an important apoptotic pathway in coronary EC under simulated ischaemia [10, 19]. Similarly, a marked cleavage of caspase-12 was found in ischaemic EC in the present study. APC nearly completely suppressed cleavage of caspase-12. In addition, APC markedly attenuated cytochrome C release from mitochondria. Both caspase-12

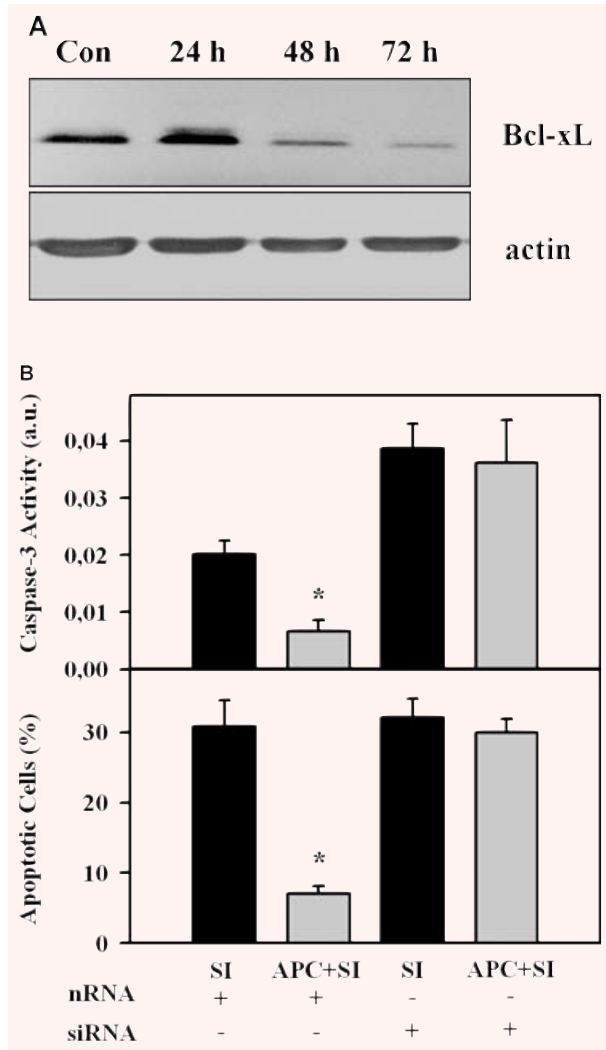


Fig. 6 (A) Western blot analysis of Bcl-xL prepared from extracts of control EC (Con) or EC after treatment with Bcl-xL targeting siRNA (siRNA) for 24, 48 and 72 hrs. Data are representative of three independent experiments with similar results. (B) Effect of pre-treatment for 72 hrs with non-targeting siRNA (nRNA) or targeting siRNA (siRNA) and acidic pre-conditioning (APC) on caspase-3 activity and the number of apoptotic EC after 2 hrs of simulated ischaemia (SI). * $P < 0.05$ versus SI + nRNA. Values are mean \pm S.E.M., $n = 4$.

and cytochrome C can lead to activation of caspase-3 [22], which activity during simulated ischaemia was significantly reduced by APC. Thus, these data suggest that APC leads to protection against ischaemic apoptosis through suppression of both ER- and mitochondria-mediated apoptotic pathways.

Extra-cellular acidosis is an important intrinsic feature of ischaemia and, therefore, may be responsible for protection

against ischaemic injury by ischaemic pre-conditioning. Indeed, comparison of ischaemic and acidic pre-conditioning performed by Lundmark and colleagues [9] on the whole heart demonstrated similar protection against necrotic injury and cardiac dysfunction. Although no reports about anti-apoptotic effect of APC are available until now, ischaemic pre-conditioning has been shown to ameliorate apoptosis during ischaemia in different tissues [23–25]. The mechanism of this anti-apoptotic effect of ischaemic pre-conditioning is still poorly understood. In line with our data, several reports suggested the suppression of the mitochondrial pathway by ischaemic pre-conditioning, indicated by a reduction of cytochrome C release [25]. A study by Hayashi *et al.* [24] also demonstrated an attenuation of ischaemia-induced ER stress in neurons, which is in agreement with the suppression of ER-bound caspase-12 cleavage in the present report.

The cellular mechanisms responsible for suppression of these two apoptotic pathways by ischaemic pre-conditioning are still debatable. Regarding delayed pre-conditioning, that is, pre-conditioning followed by a recovery period for 12–24 hrs, which is relevant to our study, an alteration in the expression of anti- and pro-apoptotic Bcl-2 family proteins [23, 26] as well as an induction of ER chaperones [24] and HSPs [16] have been demonstrated. In the present study we also tested whether APC may affect the expression of these proteins. Western blot analysis of pro-apoptotic proteins (Bax and Bak), ER-localized chaperones (GRP78 and calreticulin), HSP-27, HSP32, HSP70 and ER-bound procaspase-12 did not reveal any significant change in the steady-state expression of these proteins. In contrast, a pronounced over-expression of the anti-apoptotic protein Bcl-xL was found. In line with this finding, a previous study by Hu and Lu [23] also demonstrated over-expression of Bcl-xL, which was attributed as a key anti-apoptotic mechanism induced by ischaemic pre-conditioning. Several previous reports have also demonstrated that over-expression of this protein suppresses ER- and mitochondria-mediated apoptotic pathways [27, 28]. In agreement with these studies we found that knock-down of Bcl-xL prevented the protective effect of APC. Therefore, over-expression of Bcl-xL seems to be a key event mediating the anti-apoptotic action of APC.

The up-stream signalling responsible for the over-expression of Bcl-xL is still have to be elucidated. The transcriptional regulation of the bcl-xL gene is complex and the participating role of several transcriptional factors, for example STATs, Rel/NF- κ B, Ets and AP-1 was demonstrated [29]. Regarding the present study, a short acidic exposure has been shown to activate MAP-kinases and PI3-kinase dependent signalling pathways [30, 31], which are upstream to these transcription factors. Therefore, one could suppose, that the activation of some of these kinases may function as an up-stream mechanism induced by APC. In support of this hypothesis, a recent study of Lazou-A *et al.* [32] suggested the involvement of ERK1/2 in an anti-apoptotic effect of ischaemic pre-conditioning associated with the increased expression of Bcl-xL during ischaemia.

In conclusion, this study demonstrates for the first time that short acidic pre-treatment protects EC against ischaemia-induced

apoptosis. The mechanism of this protection consists of a suppression of ER- and mitochondria-mediated pathways. Overexpression of the anti-apoptotic protein Bcl-xL is responsible for the APC-induced resistance to apoptosis. Further investigations of the pH-sensing mechanisms responsible for this adaptation phenomenon may lead to the discovery of new therapeutic approaches for treatment of diseases accompanied by apoptosis.

Acknowledgements

The technical help of G. Scheibel and K. Rezny is gratefully acknowledged. This study was supported by Forum Grant from the Ruhr-Universität-Bochum and was part of the thesis of S. Kumar submitted in fulfillment of the requirements for the degree of Doctor of Philosophy at the Ruhr-Universität-Bochum (Germany).

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