

SLC4A7 sodium bicarbonate co-transporter controls mitochondrial apoptosis in ischaemic coronary endothelial cells

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Aims	Bicarbonate transport has been shown to participate in apoptosis under ischaemic stress. However, the precise transporting mechanisms involved in ischaemic apoptosis are unknown and were thus the aim of the present study.
Methods and results	Rat coronary endothelial cells (EC) were exposed to simulated <i>in vitro</i> ischaemia for 2 h, and apoptosis was subsequently determined by chromatin staining and caspase-3 activity analysis. By examining the expression of bicarbonate transporters (BT) in EC by reverse transcriptase polymerase chain reaction and western blotting, a marked expression of the electroneutral sodium bicarbonate co-transporter (SLC4A7) was defined. To analyse the potential role of this transporter during apoptosis, a selective inhibitor (S0859, Sanofi-Aventis) was applied. Treatment with S0859 significantly increased caspase-3 activity and elevated the number of apoptotic EC. These results were comparable with an unselective inhibition of all BT due to withdrawal of bicarbonate in the anoxic medium. Knockdown of SLC4A7 in EC by transfecting appropriate siRNA similarly increased apoptosis of EC under simulated ischaemia. The initial characterization of the participating mechanisms of SLC4A7-dependent apoptosis revealed an activation of the mitochondrial pathway of apoptosis, i.e. cleavage of caspase-9 and binding of Bax to mitochondria. In contrast, no activation of the endoplasmic reticulum-dependent pathway (caspase-12 cleavage) or the extrinsic apoptotic pathway (caspase-8 cleavage) was found. Finally, a mitochondrial localization of SLC4A7 was demonstrated.
Conclusion	The electroneutral sodium bicarbonate co-transporter SLC4A7 localizes in mitochondria and suppresses the ischaemia-induced activation of the mitochondrial pathway of apoptosis in coronary EC.
Keywords	Sodium bicarbonate co-transporter • Apoptosis • Endothelial cells • Ischaemia • Bicarbonate • Mitochondria

1. Introduction

Increasing evidence suggests that apoptosis of endothelial cells (EC) may be responsible for acute and chronic vascular diseases, e.g. by promoting atherogenesis,¹ endothelial dysfunction,² or thrombus formation.³ Myocardial ischaemia has been shown to induce endothelial apoptosis.^{4,5} Within numerous pro-apoptotic stress factors, alteration of the ion homeostasis is an important trigger of apoptosis in an ischaemic environment. Particularly, the extra- and intracellular accumulation of H⁺, which is one of the essential features during ischaemia, has been shown to be a trigger for apoptosis in various cell types,^{6–8} including coronary EC.⁹ The regulation of the

intracellular H⁺ homeostasis in EC is dependent on the activity of two ion-transporting systems: i.e. the Na⁺/H⁺ exchanger and the Na⁺- or Cl⁻-coupled bicarbonate transporters (BT).¹⁰ Although the involvement of the Na⁺/H⁺ exchanger in the regulation of apoptosis has been shown under various stress conditions, including ischaemia,⁴ the role of BT is still poorly understood. Apoptosis induced by staurosporine can be ameliorated through BT inhibition,¹¹ whereas under serum deprivation BT seems to play an anti-apoptotic role.¹² Recently, we also demonstrated an anti-apoptotic role of BT during ischaemic stress.¹³ In the present study, we thus further aimed to analyse which specific bicarbonate transporting mechanism is involved in the apoptosis signalling.

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As an experimental model, a primary culture of rat coronary EC was exposed to *in vitro* simulated ischaemia consisting of glucose-free anoxia in combination with extracellular acidosis. This model has been characterized in detail in our previous studies with respect to cytosolic Ca^{2+} and pH homeostasis and apoptotic signalling.^{9,13,14} By applying a specific inhibitor and targeted knockdown, we demonstrated that the electroneutral sodium bicarbonate co-transporter (SLC4A7) is co-localized with mitochondria and modulates the mitochondrial pathway of apoptosis during ischaemic stress.

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), and the approval for animal research was granted by the university ethic review board.

2.1 Cell culture

Coronary EC were isolated from 250–300 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.¹⁵ The purity of the cell culture (>95% EC) was confirmed by immunochemical staining with antibodies against von Willebrand factor and by uptake of acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl – 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate as previously described.¹⁶ Experiments were performed with monolayers reaching 80–90% confluence and 18 h prior to experiments the serum content in the culture medium was reduced from 20 to 5%.

2.2 *In vitro* simulated ischaemia

To simulate ischaemic conditions, cells were exposed to anoxia in combination with glucose deprivation and acidosis in a bicarbonate-buffered medium. For this purpose, cell culture medium was rapidly exchanged by a modified anoxic Tyrode's solution containing (in mmol/L): 137.8 NaCl, 2.6 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 1.2 CaCl_2 , 2.2 NaHCO_3 , pH 6.5 at 37°C. The solution was pre-gassed with 5% CO_2 –95% N_2 for 60 min. After the medium change, dishes with cultured EC were introduced into a gas-tight, temperature-controlled chamber under continuous flush with a humidified gas mixture (95% N_2 + 5% CO_2) and were incubated for 2 h at 37°C. In a separated set of experiments, a bicarbonate-free anoxic medium was used (in mmol/L): 115 NaCl, 2.6 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 1.2 CaCl_2 , 25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 6.5 at 37°C. In this experimental setup, a 100% N_2 was used to induce anoxia. Furthermore, anoxic medium containing high bicarbonate concentration (6.6 mmol/L; NaCl was appropriately reduced) was also applied as indicated. In these experiments, a mixture of 85% N_2 and 15% CO_2 was used during anoxia. In all setups, analysis after 2 h of simulated ischaemia did not reveal any significant alteration in buffer pH.

2.3 Caspase-3 activity assay

Activity of caspase-3 was detected using a colorimetric caspase-3 assay kit (Calbiochem) based on cleavage of synthetic caspase substrate-1 linked to the chromophore *p*-nitroanilide (Ac-DEVD-*p*NA). Preparation of cellular extracts and the analysis of caspase-3 activity were performed according to the manufacturer's protocol. The amount of hydrolysed substrate was measured at an optical density of 405 nm. The activity of caspase-3 was expressed in arbitrary units defined as the maximal increase in optical density, derived by linear regression, per 0.5×10^6 cells during 30 min.

2.4 Hoechst-33342 and propidium iodide staining

To distinguish between apoptotic and necrotic cells, nuclear staining with Hoechst-33342 and propidium iodide was applied as described previously.¹⁷ Briefly, cells were trypsinized, washed with phosphate-buffered saline (PBS), and incubated for 10 min with 1 $\mu\text{g}/\text{mL}$ Hoechst-33342 and 3 $\mu\text{g}/\text{mL}$ propidium iodide. The stained nuclei were visualized applying a converted fluorescence microscope with appropriate excitation (350 nm for Hoechst-33342 and 540 nm for propidium iodide) at a magnification of $\times 700$.

For quantification, a blind analysis of 200–300 nuclei from randomized four to five fields was performed. Cells were scored as apoptotic, when nuclei stained with Hoechst-33342 produced unequivocal bright blue fluorescence due to chromatin condensation/fragmentation.⁹ 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 the apoptotic population. The number of these cells did not exceed 4% of all cells.

2.5 siRNA transfection

Knockdown of SLC4A7 was achieved by treatment of EC with small interfering RNA duplexes (siRNA), corresponding to separate regions within the rat SLC4A7 RNA sequence (Accession number NM_058211, Cat. Nr: L-094808-01, Dharmacon Research Lafayette, CO, USA). In controls, non-targeting siRNA duplexes (Cat. Nr: D-001810-01) were used. Cells were transfected according to the manufacturer's instructions. Briefly, cells were seeded 1 day prior to transfection in Eagle's minimum essential medium (MEM) containing 10% foetal calf serum without antibiotics. SLC4A7 siRNA or non-targeting siRNA was mixed with oligofectamine (Invitrogen) in OptiMEM (Gibco BRL) for 15 min at room temperature and then added to culture medium with a final concentration of 60 nmol/L. Cells were then incubated at 37°C for 72 h and SLC4A7 protein expression was determined by western blot using specific antibodies, which revealed $\geq 90\%$ reduction of SLC4A7.

2.6 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) followed by treatment with DNase according to the manufacturer's instructions. About 1 μg of RNA was reverse transcribed into cDNA using the RNA PCR Core Kit (Applied Biosystems). A first cycle of cDNA synthesis of 30 min at 42°C was followed by an initial 95°C denaturation for 5 min by using oligo (dT) primers and moloney murine leukaemia virus reverse transcriptase. PCR with 10–20% of cDNA and 0.2 $\mu\text{mol}/\text{L}$ of each primer (Table 1) was performed for 34 cycles: a hot start at 95°C for 5 min, denaturation at 95°C for 40 s, annealing at 57°C for 30 s, and elongation at 72°C for 40 s, with a final extension of 10 min at 72°C. Conditions were chosen so that none of the RNAs analysed reached a plateau at the end of the amplification reaction, i.e. they were in the exponential phase of amplification. Negative PCR controls included omission of reverse transcriptase or omission of cDNA. PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed as an internal control. RT-PCR products were separated by 2% agarose gel electrophoresis and photographed under ultraviolet illumination.

2.7 Western blot

Primary antibodies were: Bax (Cell Signaling), caspase-3 (Cell Signaling), caspase-8 (Bio-Vision), caspase-9 (Stressgen), caspase-12 (recognizing the residues 100–116, Oncogene), protein disulfide isomerase (PDI; Stressgen), Tim23 (BD Transduction Laboratories), pan-cadherin (Cell Signaling), actin (Chemicon International), and SLC4A4 and SLC4A7

Table 1 Primer sequences for rat

Gene	Direction	Sequence	Product sizes (bp)
SLC4A4 (NM_053424)	Forward	ACCTTGTCCTGCACAGCT	223
	Reverse	CAGCCAGGGACCGAAGGTT	
SLC4A7 (NM_058211)	Forward	GAACAGCGGGTTACAGGGC	251
	Reverse	AGCTGGACGACCGTGAACA	
SLC4A8 (NM_199497)	Forward	CGAGCCGCTACTTCCCAAC	273
	Reverse	TGATGACGGCGGTGATCTG	
GAPDH (AF106860)	Forward	AGGGTCATCATCTCCGCCC	339
	Reverse	GCCAGTGAGCTTCCCCTTC	

(were kindly provided by Dr Praetorius from Aarhus University, Denmark). Specific bands were visualized after incubation with peroxidase-linked/HRP-labelled secondary antibodies by chemiluminescence using the ECL+ kit (Amersham Pharmacia). Equivalent sample loading was confirmed by stripping membranes with the Blot Restore Membrane Stripping Buffer (Pierce) followed by treatment with antibodies against actin.

2.8 Subcellular fractionation

Subcellular fractionation was performed as described by Heiden et al.¹⁸ with small modifications. Briefly, cells were permeabilized by resuspension in the following ice-cold buffer (in mmol/L): 210.0 sucrose, 20.0 HEPES/KOH (pH 7.5), 10.0 KCl, 1.5 MgCl₂, 1.0 EDTA, 1.0 EGTA, 1.0 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, 1:100 protease inhibitor cocktail (Sigma), and 0.04% digitonin. To sediment nuclei and debris, cells were centrifuged at ×600 g for 10 min at 4°C. The supernatant was further centrifuged at ×12 000 g for 25 min at 4°C. After this centrifugation, the pellet was used as mitochondrial fraction and the supernatant as a cytosolic fraction. The purity of mitochondrial fraction was confirmed by the absence of the endoplasmic reticulum (ER) marker, i.e. PDI, and plasmalemmal marker, i.e. cadherin.

2.9 Immunocytochemistry and confocal microscopy

Cells fixed with 4% paraformaldehyde were treated for 15 min with 0.5 mg/mL MitoTracker Red (Molecular Probes). After washing with PBS, the cells were additionally fixed for 5 min with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100 and incubated with a rabbit polyclonal anti-SLC4A7 antibody (1:200) followed by treatment with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Dianova). The cells were examined by a laser scanning confocal microscope (Leica TCS SP2). Series of confocal optical sections were taken at 0.5 μm intervals using a Leica Planapo 63/1.4 objective lens. Each recorded image was taken using dual-channel scanning and consisted of 1024 × 1024 pixels.

2.10 Statistical analysis

Data are given as mean ± SEM. The comparison of means between the groups was performed by one-way analysis of variance followed by Bonferroni *post hoc* test. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1 Pharmacological inhibition or knockdown of SLC4A7 enhances apoptosis in ischaemic EC

Since sodium-dependent bicarbonate transport plays an important role in ischaemia-induced cell injury, we examined the expression of

three widely distributed sodium BT, i.e. SLC4A4, SLC4A7, and SLC4A8, in rat coronary EC. We did not examine SLC4A5, which is expressed only in the liver, testis, and spleen.¹⁹ Applying RT-PCR analysis, we found a marked expression of SLC4A7, a slight expression of SLC4A4, and no expression of SLC4A8 in our cells (Figure 1A). A subsequent expression analysis of the sodium bicarbonate co-transporters on the protein level by western blot revealed only expression of SLC4A7 in coronary EC (Figure 1B).

To examine the contribution of SLC4A7 in ischaemia-induced apoptosis of EC, an inhibitor of SLC4A7, substance S0859 (kindly provided by Dr Kleemann, Sanofi-Aventis), was applied. This compound was shown to be a selective inhibitor for sodium bicarbonate co-transporter and displays no effects on the activity of carbonic anhydrase, chloride–bicarbonate exchangers, and the sodium–proton exchanger.²⁰ Treatment with 1 μmol/L S0859 had no effect on apoptosis in control normoxic cells, but significantly increased the apoptotic cell number and the caspase-3 activity during ischaemic stress (Figure 2). Importantly, an unspecific suppression of all BT by omission of bicarbonate during ischaemic incubation (HEPES-buffered medium) revealed similar effects. In opposite, an increase of the bicarbonate concentration in the anoxic medium (6.6 vs. 2.2 mmol/L) significantly suppressed the caspase-3 activity and reduced the number of apoptotic cells.

To further substantiate the findings with S0859, a specific knockdown of SLC4A7 by transfection with siRNA was performed. Treatment with siRNA induced an about 90% suppression of SLC4A7 expression as analysed by western blot, whereas no effect could be detected using non-targeting siRNA (Figure 3A). Knockdown of SLC4A7 led to a significant rise in apoptotic cell number and caspase-3 activity in ischaemic, but not in control cells (Figure 3B). Again, no effects could be detected after treatment with non-targeting siRNA. Thus, selective SLC4A7 knockdown demonstrates an important role of this transporter in modulating the ischaemia-induced apoptosis of EC.

Although we did not find SLC4A7 expression on the protein level by western blot, its slight expression could be detected by RT-PCR analysis. Therefore, to prove a possible contribution of SLC4A4 to apoptosis, we combined SLC4A7 knockdown with S0859 treatment, which is a generic sodium bicarbonate co-transporter inhibitor.²⁰ We found that the combination of SLC4A7 knockdown with S0859 treatment had a similar effect on the EC apoptosis compared with SLC4A7 knockdown alone (Figure 3B).

Although simulated ischaemia slightly but significantly increased the number of necrotic cells, none of the applied treatments affected the rate of necrotic cell death (Figures 2 and 3B).

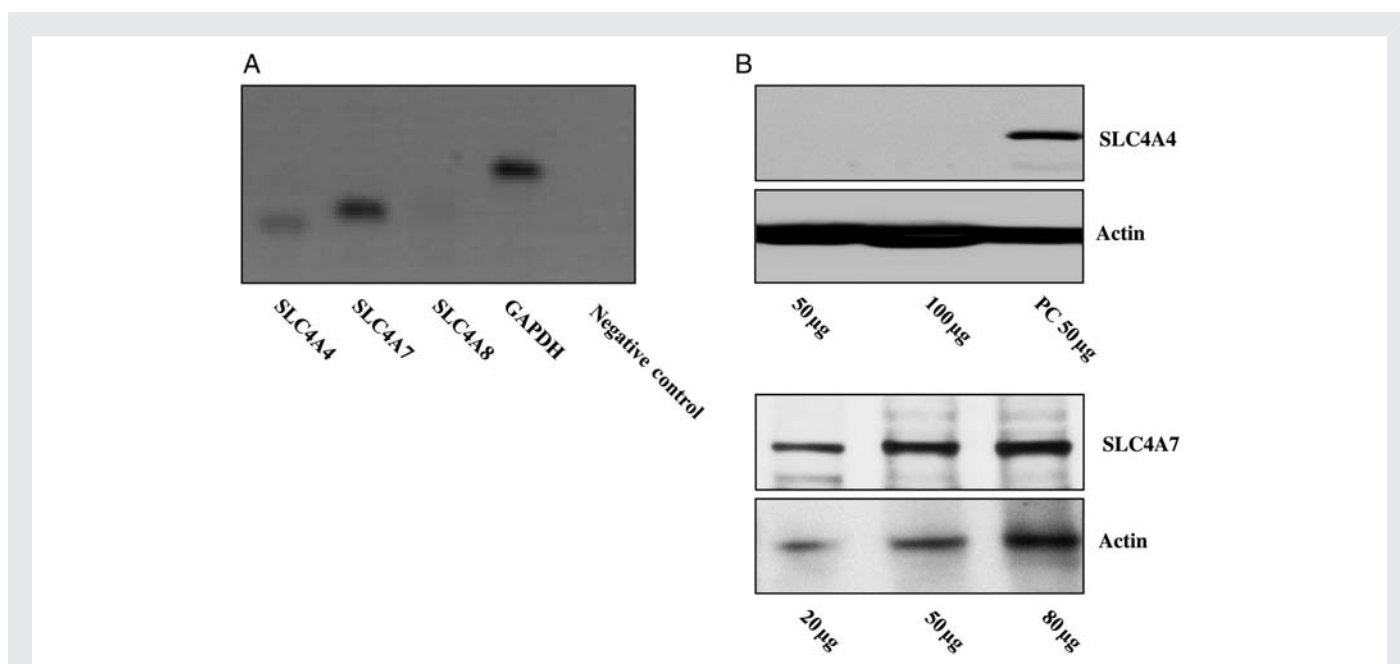


Figure 1 SLC4A7 is markedly expressed in coronary EC. (A) RT-PCR analysis of SLC4A4, SLC4A7, and SLC4A8 transcripts in rat coronary EC. GAPDH was used as internal control. (B) Western blot analysis of SLC4A4 and SLC4A7 expression in rat coronary EC. Rat kidney lysate was used as a positive control (PC) for SLC4A4. Amount of protein loaded in micrograms per well is indicated. Data are representative from four to five independent experiments with similar results

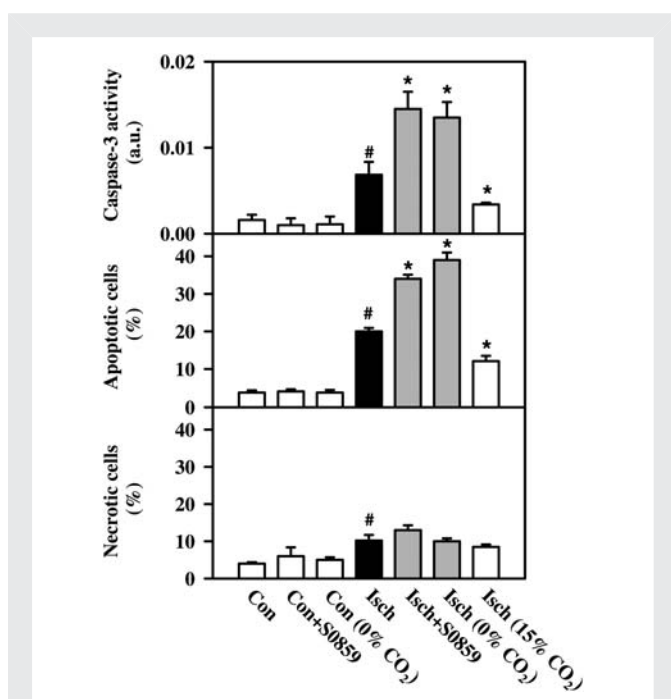


Figure 2 Caspase-3 activity and numbers of apoptotic and necrotic EC under control conditions (Con) or after 2 h of simulated ischaemia in bicarbonate-buffered medium (Isch) in the absence or presence of SLC4A7 inhibitor S0859 (1 µmol/L). Additionally, ischaemia was performed in bicarbonate-free, HEPES-buffered medium (0% CO₂) or in medium with elevated bicarbonate concentration (6.6 mmol/L, 15% CO₂). Values are mean ± SEM, n = 8–10. #P < 0.05 vs. Con.; *P < 0.05 vs. Isch.

3.2 SLC4A7 specifically controls the mitochondrial pathway of apoptosis

To find out which apoptotic pathway is activated by inhibition of SLC4A7, we analysed the effects of S0859 treatment on the death receptor pathway (caspase-8 cleavage), the ER pathway (caspase-12 cleavage), and the mitochondrial pathway (caspase-9 cleavage). Treatment with S0859 had no effects on caspase-8 and caspase-12 cleavage (Figure 4A–C). In contrast, inhibition of SLC4A7 significantly increased caspase-9 cleavage suggesting that SLC4A7 participates in the mitochondrial pathway of apoptosis. Similarly, knockdown of SLC4A7 aggravated caspase-9 cleavage during simulated ischaemia (Figure 4D and E). Both S0859 treatment and SLC4A7 knockdown markedly increased cleavage of the effector protein caspase-3, which is in agreement with the observed rise of caspase-3 activity demonstrated in Figures 2 and 3B.

Translocation of pro-apoptotic members of the Bcl-2 family proteins, for example Bax, from the cytosol to mitochondria is an important initial mechanism for the activation of the mitochondrial pathway of apoptosis.²¹ In the present study, we tested whether SLC4A7 inhibition may lead to a mitochondrial Bax translocation. For this purpose, a western blot analysis of the Bax protein in the mitochondrial fraction was performed at the end of simulated ischaemia. We found that simulated ischaemia in bicarbonate-buffered medium slightly increased mitochondrial Bax localization (Figure 4D). This Bax binding to mitochondria was significantly augmented by targeting siRNA treatment. Thus, under simulated ischaemia, translocation of Bax to mitochondria is dependent on SLC4A7 activity.

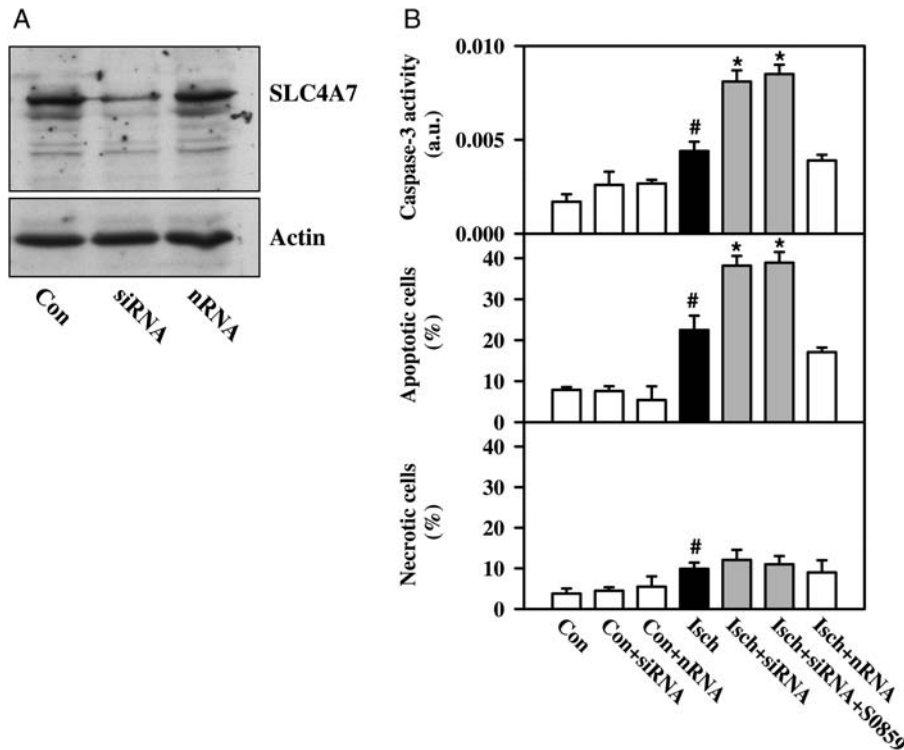


Figure 3 Knockdown of SLC4A7 aggravates ischaemia-induced apoptosis of EC. (A) Western blot of SLC4A7 prepared from extracts of EC after treatment with targeting (siRNA) or non-targeting (nRNA) siRNA for 72 h. Data are representative from five independent experiments with similar results. (B) Knockdown of SLC4A7 by treatment with siRNA increased caspase-3 activity and apoptotic cell number under ischaemic stress (Isch), but not under control conditions (Con). No significant effects were found after treatment with non-targeting siRNA (nRNA). Combined treatment with siRNA and S0859 had no effect on apoptosis compared with siRNA-treatment alone. Values are mean \pm SEM, $n = 6-10$. [#] $P < 0.05$ vs. Con.; ^{*} $P < 0.05$ vs. Isch.

3.3 SLC4A7 is co-localized with mitochondria

Until now, no data were available regarding a co-localization of SLC4A7 with mitochondria, but the participation of SLC4A7 in the regulation of the mitochondrial pathway of apoptosis suggests a mitochondrial localization of this transporter. Western blot analysis of SLC4A7 revealed a marked presence of SLC4A7 in the purified mitochondrial fraction (Figure 5). The purity of mitochondrial fraction was confirmed by the absence of PDI (ER marker) and cadherin (plasma-membral marker). Furthermore, applying immunocytochemistry, we demonstrated a co-localization of SLC4A7 with mitochondria in coronary EC (Figure 6). No mitochondrial co-localization of SLC4A7 was observed after SLC4A7 knockdown, as demonstrated by western blot (Figure 5C) or immunocytochemistry (Figure 6) analyses.

4. Discussion

The aim of the present study was to elucidate which bicarbonate transporting mechanism is involved in the ischaemia-induced apoptosis of coronary EC. The main findings are as follows: (i) SLC4A7 is markedly expressed in coronary EC; (ii) selective pharmacological inhibition as well as knockdown of SLC4A7 aggravated apoptosis in ischaemic EC due to activation of the mitochondrial pathway of apoptosis; (iii) analysis of the intracellular distribution of SLC4A7 revealed a co-localization of this transporter with mitochondria.

Glucose-free anoxia was combined with extracellular acidosis (pH 6.4) to simulate ischaemia in the present study. This model of ischaemia was characterized in our previous study, which demonstrated the importance of cytosolic acidification as an initial trigger for Ca^{2+} release from the ER followed by the cleavage of the ER-bound caspase-12.⁹ Surprisingly, the mitochondrial pathway of apoptosis in coronary EC plays, however, only a minor role in the simulated ischaemia-induced apoptosis. Nevertheless, applying ischaemia in bicarbonate-free, HEPES-buffered medium or treatment with an unselective inhibitor of BT markedly activated the mitochondrial pathway of apoptosis.¹³ Thus, it seems that BT plays an important role in the mitochondrial pathway of apoptosis in coronary EC. Which specific bicarbonate transporting mechanisms are responsible for such results was unknown.

In this study, we found that within the three sodium-coupled BT, the electroneutral sodium bicarbonate co-transporter (SLC4A7) is markedly expressed in coronary EC. Applying two different tools to suppress SLC4A7 activity, i.e. pharmacological inhibition with the selective inhibitor S0859²⁰ or targeted knockdown, we found a significant aggravation of ischaemia-induced apoptosis. Although we did not analyse the role of other sodium-independent BT, the finding that selective suppression of SLC4A7 induced a rise of apoptotic rate similar to those observed under unselective inhibition of total bicarbonate transport in EC by bicarbonate omission (Figure 2) suggests that SLC4A7 is the key BT mediating apoptosis in ischaemic EC.

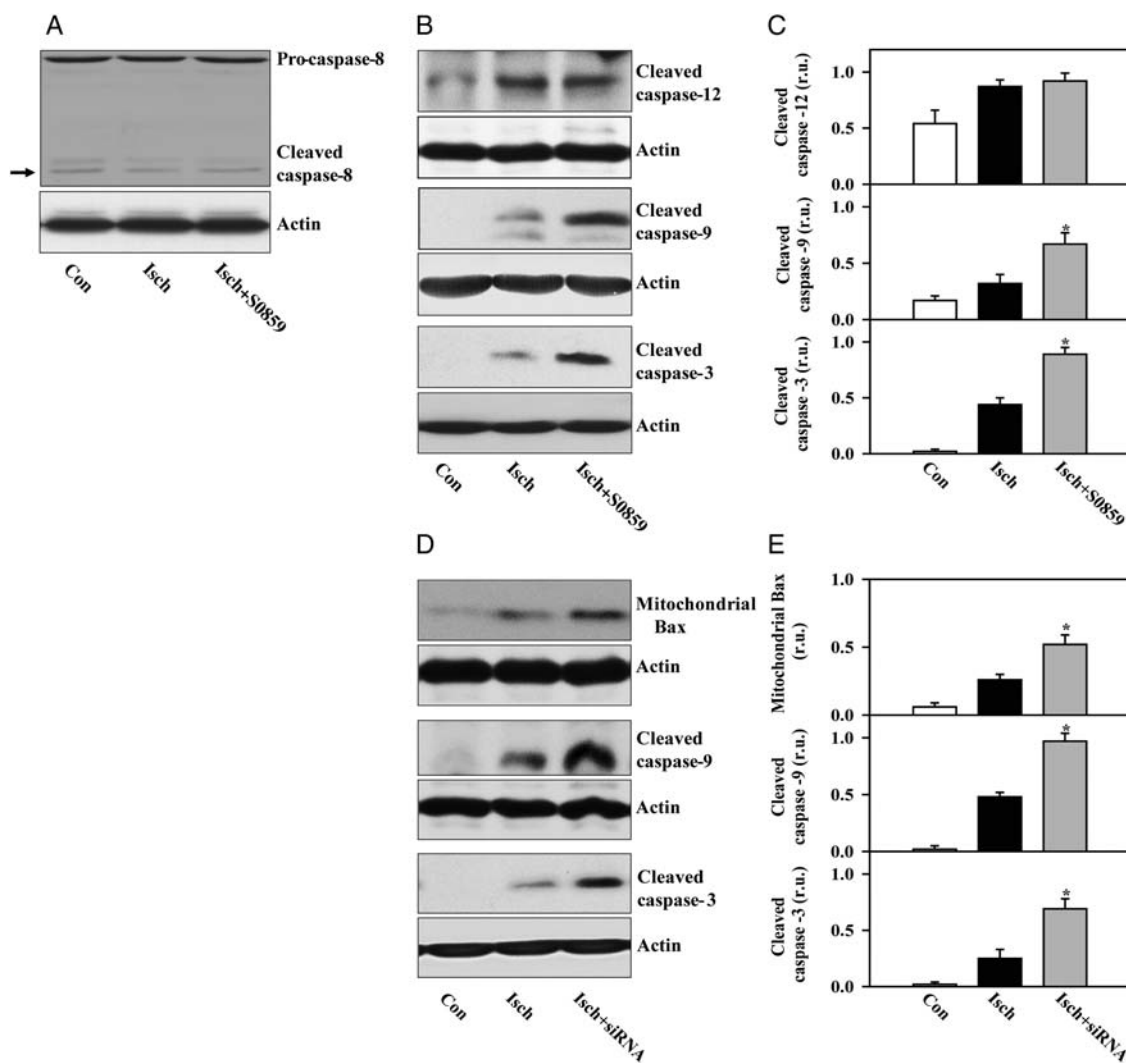


Figure 4 SLC4A7 controls the mitochondrial pathway of apoptosis. (A and B) Western blot analysis of cleaved caspase-8, -12, -9, and -3 prepared from lysates of control EC or EC exposed to ischaemia without (Isch) or under treatment with 1 μ mol/L S0859, an inhibitor of SLC4A7 (Isch+S0859). Arrow indicates 41 kDa cleaved caspase-8 bands. (C) Statistical analysis of band density of cleaved caspase-12, -9, and -3 expressed as a ratio to the own actin band density (relative units). (D) Western blot analysis of mitochondrial Bax and cleaved caspase-9 and -3 prepared from lysates of control EC or EC exposed to ischaemia without treatment (Isch) or after SLC4A7 knockdown (Isch+siRNA). (E) Statistical analysis of band density of mitochondrial Bax and cleaved caspase-9 and -3 expressed as a ratio to the own actin band density (relative units). All western blot data are representative of five to six independent experiments with similar results. Values are mean \pm SEM, $n = 5-6$. * $P < 0.05$ vs. Isch.

In line with the present data, a few recent reports demonstrated that unspecific BT inhibition promotes cell death.^{11,22} Importantly, a previous study of Bok *et al.*²³ applying SLC4A7-knockout mice demonstrated that the absence of this transporter promotes apoptosis of photoreceptors leading to mice blindness. Unfortunately, the cellular mechanism leading to SLC4A7-dependent apoptosis was not analysed in this study.

To find out which apoptotic pathways were activated by SLC4A7 inhibition, cleavage of caspase-8, caspase-12, and caspase-9 was investigated in the present study. Activation of the death receptor pathway is unlikely responsible for the pro-apoptotic effect of the SLC4A7 inhibition, since no caspase-8 cleavage could be detected under ischaemia without or in the presence of an SLC4A7 inhibitor. Cleavage of the ER-bound caspase-12 was previously reported to be a main apoptotic pathway in coronary EC during simulated ischaemia.⁹

As expected, a marked cleavage of caspase-12 was found in ischaemic EC in the present study. However, inhibition of SLC4A7 had no effect on caspase-12 cleavage. In contrast, cleavage of caspase-9 was markedly augmented by pharmacological SLC4A7 inhibition or by targeted knockdown. Furthermore, SLC4A7 knockdown led to a significant increase of Bax binding to mitochondria, which is an important mechanism triggering the mitochondria-dependent apoptosis.^{20,24} Thus, these findings strongly suggest that inhibition of SLC4A7 in ischaemic EC lead to an activation of the mitochondrial pathway of apoptosis.

How SLC4A7 may control the mitochondrial pathway of apoptosis is still obscure. Plasmalemmal SLC4A7 together with the Na⁺/H⁺ exchanger play an essential role in regulating cytosolic pH in EC.¹⁰ Regarding ischaemia, one could argue that inhibition of plasmalemmal BT may lead to an enhanced cytosolic acidification, which in EC is an important trigger for cytosolic Ca²⁺ overload.⁹ Excessive

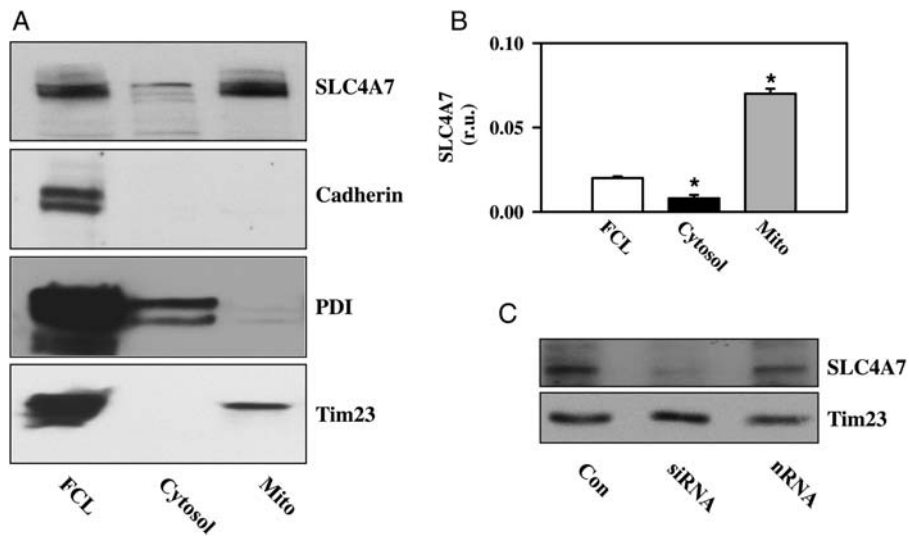


Figure 5 (A) Western blot analysis of SLC4A7, cadherin (plasmalemma marker), PDI (endoplasmic reticulum marker), and Tim23 (mitochondrial marker) prepared from different subcellular fractions of EC. FCL, full cell lysates (50 μ g protein per well); Cytosol, cytosolic fraction (50 μ g protein per well); Mito, mitochondrial fraction (10 μ g protein per well). (B) Statistical analysis of band density of SLC4A7 (Figure 5A) expressed as a ratio to the amount of loaded proteins (relative units). Values are mean \pm SEM, $n = 5$. * $P < 0.05$ vs. FCL. (C) Western blot analysis of SLC4A7 in mitochondrial fractions of control EC or in cells treated with targeting (siRNA) or non-targeting (nRNA) siRNA. All western blot data are representative of five independent experiments with similar results.

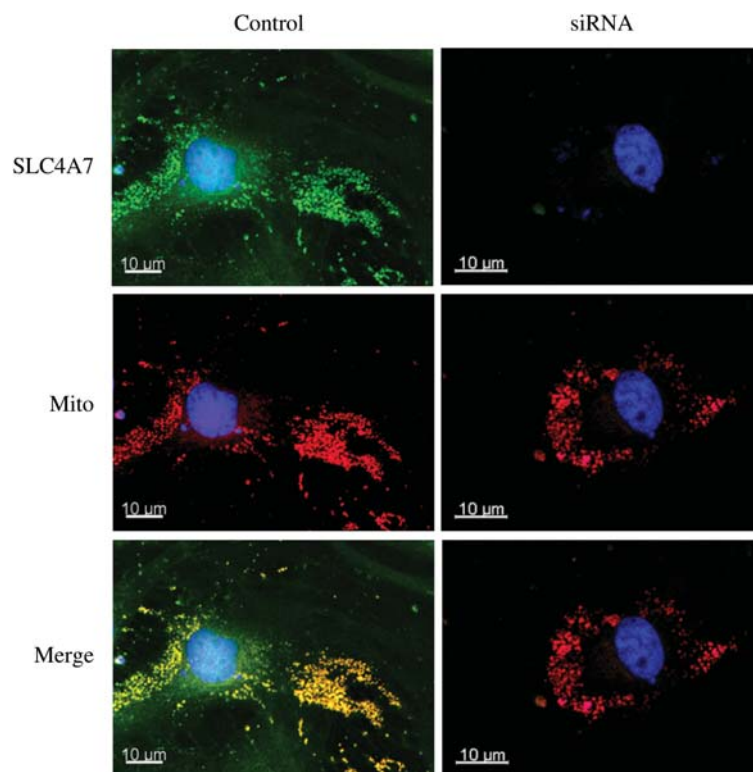


Figure 6 Immunostaining analysis of mitochondrial SLC4A7 localization in untreated (Control) or SLC4A7 siRNA-treated (siRNA) EC. SLC4A7 was labelled with a specific antibody (green) and mitochondria with MitoTracker (red). Nuclei are stained blue with 4',6-diamidino-2-phenylindole. Yellow–orange spots in the merge image represent the co-localization of SLC4A7 with mitochondria. Data are representative from four independent experiments with similar results.

accumulation of Ca^{2+} in the cytosol in turn can jeopardize the mitochondrial function and promote apoptosis.²⁵ However, analysis of the cytosolic ion homeostasis in our previous study revealed that unselective inhibition of BT in EC under simulated ischaemia had no effect on cytosolic acidosis and rather slightly reduced Ca^{2+} overload.¹³ Thus, aggravation of ischaemic apoptosis under SLC4A7 inhibition is unlikely due to the alteration in cytosolic Ca^{2+} and H^+ homeostasis.

Besides the plasmalemmal BT, a potential contribution of mitochondria-localized BT in the mitochondrial pathway of apoptosis can be supposed. Although a few previous reports provided evidence for a bicarbonate flux across the mitochondrial membrane,^{26,27} no data were available regarding the precise bicarbonate transporting systems in mitochondria. In the present study, a distinct co-localization of SLC4A7 with mitochondria was found. This finding suggests that specifically the mitochondria-localized SLC4A7 may be responsible for controlling the mitochondrial pathway of apoptosis.

How mitochondria-localized SLC4A7 may suppress the mitochondrial pathway of apoptosis under ischaemic stress remains unresolved. Several previous reports suggested that ischaemia-induced cytosolic acidification results in acidification of mitochondrial matrix.^{28,29} Such an alteration of the matrix pH has been shown to contribute in mitochondrial swelling,²⁹ which may induce cytochrome C release. It is still unknown whether BT may contribute to the regulation of matrix pH under ischaemic stress. One could suppose that intra-mitochondrial, SLC4A7-dependent HCO_3^- influx (due to sodium gradient³⁰) may lead to an attenuation of matrix acidification, thus preventing mitochondrial swelling.

Alternatively, the influx of bicarbonate anions into mitochondria may lead to the release of CO_2 as a result of carbonic acid degradation. It has been suggested that CO_2 reacting with peroxynitrite may scavenge this highly reactive radical.³¹ Since peroxynitrite may trigger the mitochondrial pathway of apoptosis,³² scavenging of peroxynitrite by CO_2 may, thus, interfere with apoptosis in our model. On the other side, reaction of CO_2 with peroxynitrite leads to the formation of carbonate radicals. In recent years, the carbonate radical has been proposed to be a key mediator of the oxidative damage resulting from peroxynitrite production, xanthine oxidase turnover, and superoxide dismutase-1 peroxidase activity.³³ In an aqueous environment, the carbonate radical as a charged species has been suggested to be a strong oxidizing agent leading to oxidation of proteins and nucleic acid. Furthermore, in contrast to hydroxyl radicals, carbonate radicals are able to diffuse longer distances.³⁴ Thus, carbonate radicals may significantly contribute to the translation of oxidative signal from the origin of oxygen radicals, e.g. mitochondria, to distant targets within the cell. Whether carbonate radicals may contribute to mitochondrial damage and apoptosis is still unknown.

Aside of CO_2 -dependent modulation of oxidative stress, a mitochondrial HCO_3^- influx may lead to the activation of bicarbonate-sensitive targets, like soluble adenylyl cyclase. Indeed, this cyclase may control the activity of the cytochrome C oxidase and the oxygen radical production through the cAMP/protein kinase A pathway.³⁵ Reduction of the bicarbonate influx may suppress the mitochondrial adenylyl cyclase activity followed by an increase in mitochondrial oxygen radical production, which is an important trigger of apoptosis.

In contrast to widely accepted pro-apoptotic action of oxygen radicals, several previous reports suggest an anti-apoptotic effect of oxygen radicals due to activation of the protein kinase B (Akt) and

mitogen-activated protein (MAP) kinase signalling.³⁶ Indeed, in our model during progression of hypoxia to anoxia at the beginning of simulated ischaemia, the oxygen radical formation at complex III in mitochondria may occur.^{37,38} Nevertheless, such oxygen radical-dependent activation of Akt and MAP kinases unlikely contributes to apoptosis in our model. In a previous study, applying a similar model of simulated ischaemia in coronary EC,³⁹ we found that suppression of Akt or p38 kinase has no effect on the ischaemia-induced apoptosis.

In conclusion, this study demonstrates for the first time the presence of SLC4A7 in mitochondria and postulates its role in mediating the mitochondrial pathway of apoptosis during ischaemic stress. Further investigation of the underlying mechanisms of SLC4A7 action may potentially contribute to the discovery of new therapeutic approaches for the treatment of diseases accompanied by resistance to apoptosis, for example, cancer and neurodegenerative diseases.

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