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Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning☆

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Abstract

Objective: Connexin 43 (Cx43) is involved in infarct size reduction by ischemic preconditioning (IP); the underlying mechanism of protection, however, is unknown. Since mitochondria have been proposed to be involved in IP's protection, the present study analyzed whether Cx43 is localized at mitochondria of cardiomyocytes and whether such localization is affected by IP.
Methods and results: Western blot analysis on mitochondrial preparations isolated from rat, mouse, pig, and human hearts showed the presence of Cx43. The preparations were not contaminated with markers for other cell compartments. The localization of Cx43 to mitochondria was also confirmed by FACS sorting (double staining with MitoTracker Red and Cx43) and immuno-electron and confocal microscopy. To study the role of Cx43 in IP, mitochondria were isolated from the ischemic anterior wall (AW) and the control posterior wall (PW) of pig myocardium at the end of 90 min low-flow ischemia without ($n=13$) or with ($n=13$) a preceding preconditioning cycle of 10 min ischemia and 15 min reperfusion. With IP, the mitochondrial Cx43/adenine nucleotide transporter ratio was 3.4 ± 0.7 fold greater in AW than in PW, whereas the ratio remained unchanged in non-preconditioned myocardium (1.1 ± 0.2, $p<0.05$). The enhancement of the mitochondrial Cx43 protein level occurred rapidly, since an increase of mitochondrial Cx43 was already detected with two cycles of 5 min ischemia/reperfusion in isolated rat hearts to $262\pm63\%$ of baseline.
Conclusion: These data demonstrate that Cx43 is localized at cardiomyocyte mitochondria and that IP enhances such mitochondrial localization.

Keywords: Connexin; Mitochondria; Preconditioning

This article is referred to in the Editorial by S. Dhein (pages 179–181) in this issue.

1. Introduction

Transient periods of ischemia/reperfusion delay the development of cell death from a subsequent prolonged episode of ischemia/reperfusion. The underlying molecular mechanism of this protection, known as ischemic preconditioning (IP), remains to be elucidated in detail [1,2].

The gap junction protein connexin 43 (Cx43) is highly expressed in cardiomyocytes (for review, see [3]), and is involved in IP, since uncoupling prevents IP in mice [4]. Furthermore, hearts and isolated cardiomyocytes from

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heterozygous Cx43-deficient mice cannot be preconditioned [5]. Isolated cardiomyocytes from wildtype mice can be preconditioned [6]. Thus, Cx43 at the single cardiomyocyte level is important for IP and this excludes a role for cell–cell communication in IP.

Although it is generally assumed that Cx43 is exclusively localized at the sarcolemma, there is some evidence that this may not be the case. It has been recently shown that the carboxy-terminus of the protein can also be localized at cardiomyocyte nuclei [14]. In cultured human endothelial cells, Cx43 was also detected in mitochondria, where its level increased in response to cellular stress [15].

Since mitochondria play an important role in triggering cardioprotection and have been suggested to act as an end-effector of IP [7–13], the aims of the present study were (1) to investigate whether Cx43 is localized at cardiomyocyte mitochondria, and (2) whether the mitochondrial content of Cx43 is affected by IP. Therefore, mitochondria were isolated from non-preconditioned mouse, rat, pig and human hearts, as well as from preconditioned rat and pig hearts and analyzed by Western blot, confocal microscopy, FACS (fluorescence-assisted cell sorting), and immuno-electron microscopy.

2. Methods

The present study was performed with approval by the Bioethical Committee of the district of Düsseldorf, Germany and by the Ethics Committee on Animal Research of the Hospital Vall d'Hebron, Barcelona, Spain. It 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).

2.1. Cardiomyocytes

Mouse HL-1 atrial myocytes were cultured until 70–80% confluence as previously described [16]. Pig cardiomyocytes were isolated from the left ventricle as already described [17].

2.2. Human myocardial samples

Human left ventricular tissue was obtained from patients with obstructive hypertrophic cardiomyopathy and was analyzed for mitochondrial Cx43 content by Western blot and confocal laser scan microscopy.

2.3. Rat hearts in vitro

Adult male Sprague–Dawley rats were killed by an intraperitoneal overdose of pentobarbital. The hearts were quickly removed and retrogradely perfused with oxygenated Krebs solution as previously described [18]. Four hearts were submitted to 20 min of normoxic perfusion whereas four additional preparations were submitted to two preconditioning cycles of 5 min of global ischemia followed by 5 min of reperfusion (ischemic preconditioning cycles).

2.4. Mouse hearts in vivo

The ablation of Cx43 was induced in adult $Cx43^{Cre\text{-}ER(T)/fl}$ mice by the intraperitoneal injection of 3 mg 4-hydroxytamoxifen (4-OHT) once per day on five consecutive days as previously described [19]. The animals were sacrificed at day 12 after the first injection. For control, untreated $Cx43^{Cre\text{-}ER(T)/fl}$ or $Cx43^{fl/fl}$ mice were used. The right ventricles were used for total protein extraction, the left ventricles for mitochondria isolation ($n=3$ in each group).

2.5. Pig hearts in vivo

For details of the pig preparation, see [20]. Göttinger minipigs were initially sedated (1 g ketamine hydrochloride intramuscularly), and then anesthetized with thiopental (Trapanal, 500 mg, intravenously). The anesthesia was maintained using enflurane (1–1.5%, oxygen/nitrous oxide mixture 40:60%). Following surgical instrumentation, the minipigs underwent 90 min low-flow ischemia without ($n=13$) or with ($n=13$) a preceding preconditioning cycle of 10 min ischemia and 15 min reperfusion. Tissue samples were collected from the anterior and posterior myocardium of pigs at the end of the 90-min ischemic period. In a subset of pigs from each group ($n=6$ without IP, $n=7$ with IP), reperfusion was initiated for 120 min to allow quantification of infarct size by 2,3,5-triphenyl tetrazolium chloride (TTC) staining. In those pigs, regional myocardial blood flow was assessed at baseline and at 5 min ischemia using radiolabeled microspheres [20].

2.6. Mitochondria isolation

Cardiac mitochondria were isolated from rat hearts as previously described [16] using a modification of the protocol described by Holmuhamedov et al. [21].

Mitochondria from mouse, pig and human hearts were isolated using standard centrifugation techniques [22] and purified by 30% percoll gradient ultracentrifugation. The purification procedure was assessed as the enrichment in mitochondrial proteins as well as the elimination of other cellular constituents by means of Western blot analysis.

2.7. Flow cytometry and FACS

Isolated rat heart mitochondria were incubated in 1 μM MitoTracker Red CMXRos (Molecular Probes), a specific mitochondria-selective dye, for 30 min (37 °C), subsequently, the excess of tracer was removed by washing. MitoTracker Red labeled mitochondrial fraction was incu-

bated for 1 h with anti-Cx43 antibody (dilution 1:200) at 37 °C, washed and detected with Alexa-488 conjugated secondary antibody (Molecular Probes). Negative control was obtained omitting primary antibody. Labeled mitochondria were identified on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) on the basis of Forward and Sideward Scatter parameters and red fluorescence. Forty thousand events from each sample were analyzed, gating those according with mitochondrial criteria (about 18000 events/sample). Cx43 fluorescence was detected with a 530/30 filter, and then analyzed with Cell Quest software (BD Biosciences).

Mitochondrial fractions to be used for inmunoblotting analysis were further purified by FACS in a BD FACSaria Cell Sorter (BD Biosciences). Only those events positive to MitoTracker Red were selected for separation and collection using the analysis software BD FACSDiva. About 5 to 6 millions of mitochondria were obtained per 0.3 g of ventricular tissue.

As a positive control and reference for Cx43 content in mitochondrial preparations, intercalated disk fractions were obtained from rat hearts by a modification of the method described by Miura et al. [23].

2.8. Immuno-electron microscopy

Tissue samples of rat hearts previously submitted to 20 min of normoxia or cycles of preconditioning ischemia/reperfusion were embedded in paraformaldehyde following routine embedding procedures. Cryosections (45 nm) were prepared, stained with anti-Cx43 antibody and subsequently with protein A-conjugated colloidal gold (EM Lab., Dr. Slot Utrecht University). The cryosections were examined with a Jeol JEM 1010 transmission electron microscope. As negative control, the primary antibody was omitted.

2.9. Immunohistochemistry

Purified pig mitochondria or fixed and permeabilized HL-1 cells were incubated with anti-Cx43 and anti-cytochrome *c* or anti-Ox-Phos complex II antibodies and, after washing, with the respective secondary antibodies. Mitochondria or cells were examined by confocal laser scan microscopy (Pascal, Zeiss, Jena, Germany, or Leica TCSSP2AOBS, Wetzlar, Germany) at 630× magnification.

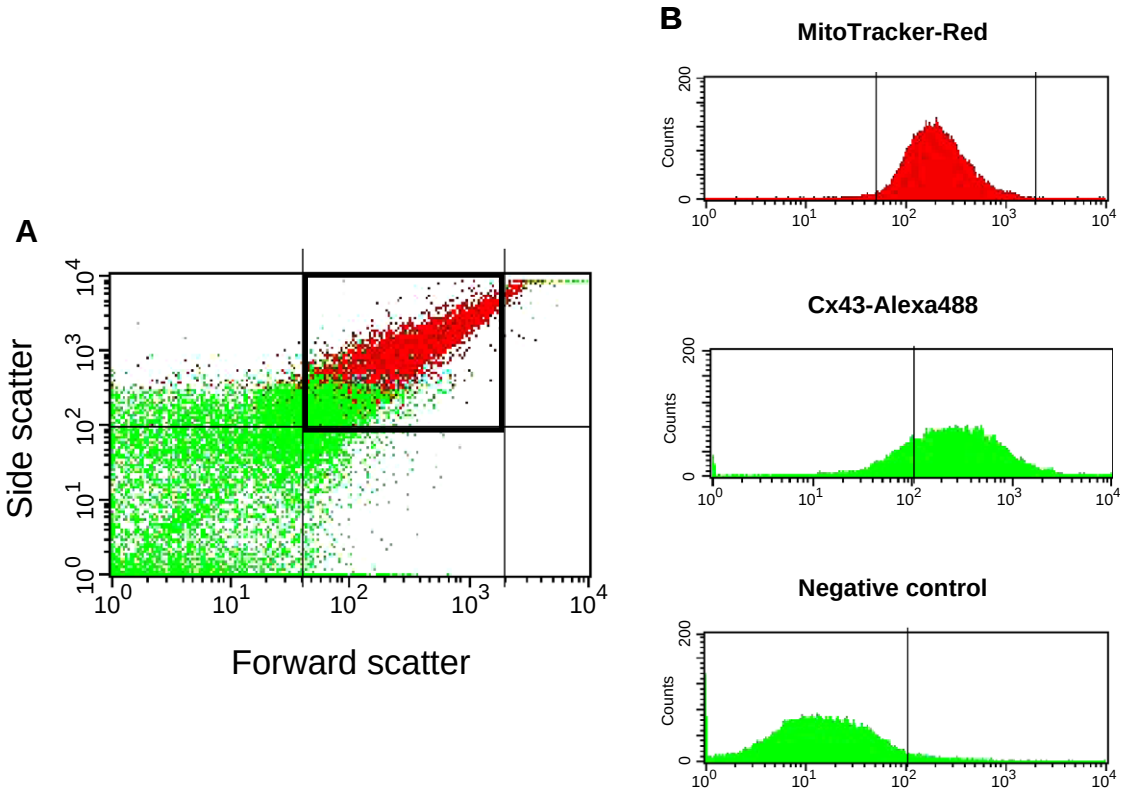

Fig. 1. FACS analysis. (A) Scatter dot plot of isolated rat mitochondria double stained for MitoTracker Red and Cx43. (B) Upper panel: MitoTracker positive events were selected for further Cx43-Alexa488 fluorescence analysis. Middle panel: Fluorescence histogram from Cx43-Alexa488 labeled MitoTracker-selected mitochondria from rat heart. Events under 10^2 fluorescence units were considered as negative (laser output was adjusted to include 1% of events in non-stained mitochondria). Lower panel: Fluorescence histogram in negative control mitochondria (primary antibody omitted). Both the number of positive events (95% vs. 15%) and mean fluorescence (411 vs. 31 fluorescence units) demonstrate the presence of Cx43 in a vast proportion of mitochondria ($p < 0.001$ for both).

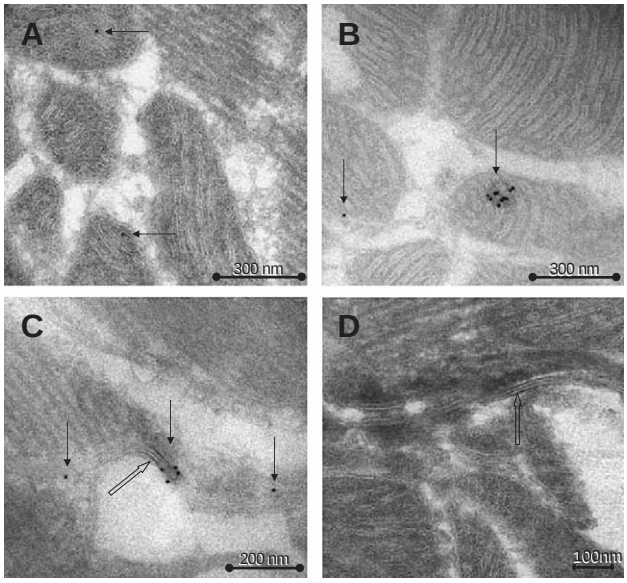

Fig. 2. Representative images of immuno-electron labeling of Cx43 in cardiomyocytes from control (A) and preconditioned (B) rat hearts. 10 nM colloidal gold labeling was associated with mitochondrial cristae. Panels C and D show gap junction zones as positive (C), and negative controls (D). In D, the primary antibody was omitted.

2.10. Western blot

Western blot analysis on mitochondrial proteins was performed according to standard procedures [24]. 10–40 μg protein extracts were electrophoretically separated on 10% polyacrylamide gels. Immunoreactive bands were detected using the SuperSignal West Femto Maximum Sensitivity Substrate or the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) and densitometrically quantified.

2.11. Antibodies

The following antibodies were used: rabbit polyclonal anti-rat total connexin43 (no. 71-0700, Zymed, Berlin, Germany, dilution of 1:1000 for Western blot (WB), 1:50 for confocal microscopy, 1:200 for FACS analysis, 1:20 for electron microscopy), mouse monoclonal anti-rat non-phosphorylated connexin43 (no. 13-8300, Zymed, Berlin, Germany, dilution of 1:750 for WB), mouse monoclonal anti-rat sodium/potassium (Na^+/K^+)-ATPase (Upstate, Waltham, MA, 1:10 000 for WB), mouse monoclonal anti-dog sarcoplasmic calcium (SERCA2)-ATPase (Sigma, Saint Louis, MO, dilution of 1:2500 for WB), goat polyclonal anti-human adenine nucleotide transporter (ANT, Santa Cruz, Santa Cruz, CA, dilution of 1:100 for WB), rabbit polyclonal anti-human Gαs (Santa Cruz, dilution of 1:500 for WB), mouse monoclonal anti-human CD31 (Alexis, Grünberg, Germany, dilution of 1:100 for WB), mouse monoclonal anti-human cytochrome *c* (DPC Biermann, Bad Nauheim, Germany, dilution of 1:50 for confocal microscopy), mouse monoclonal anti-rabbit GAPDH (Hytest, Turku, Finland, dilution of 1:2500 for WB) and mouse anti-human succinate-ubiquinol oxidoreductase (Ox-Phos Complex II, Molecular Probes, Eugene, OR, dilution of 1:2500 for WB, dilution of 1:1000 for FACS and confocal microscopy).

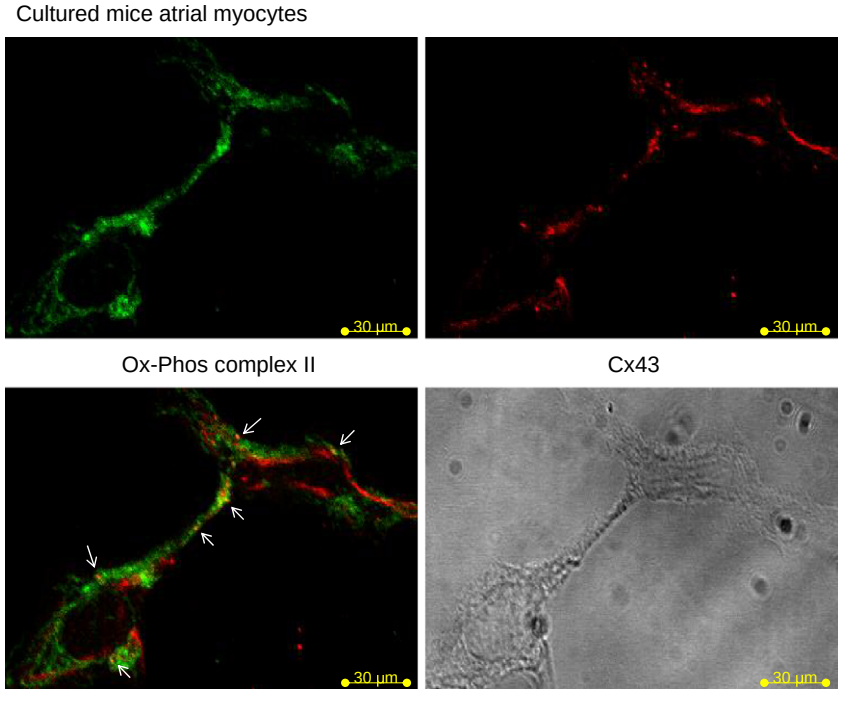

Fig. 3. Cultured mice atrial HL-1 cardiomyocytes were stained with antibodies against Cx43 (red) and mitochondria (Ox-Phos Complex II, green) and analyzed by confocal laser scan microscopy. Merged image demonstrates co-localization as yellow points (arrows). Additionally, the phase contrast image is shown.

2.12. Statistics

Data are presented as mean values ± S.E.M. Heart rate, left ventricular (LV) pressures, the maximum derivative of LV pressure (LV dP/dt_{max}), coronary arterial pressure and coronary blood flow over the time course of the protocol in the two groups of pigs were compared using two-way ANOVA. Similarly, heart rate and LV pressure over the time course in the two groups of rats were compared by two-way ANOVA. All other data were compared between non-preconditioned and preconditioned hearts by two-sided unpaired Student's *t*-test. Results were considered statistically significant at a p value <0.05.

3. Results

3.1. Localization of Cx43 at mitochondria

Flow cytometry of MitoTracker Red and Cx43 labeled mitochondrial preparations obtained from rat hearts demonstrated particles with the size within the range characteristic of mitochondria. Almost all particles stained positive for MitoTracker Red also had staining for Cx43 (Fig. 1).

Immuno-electron analysis of myocardial samples from left ventricular rat myocardium demonstrated the presence of colloidal gold-marked Cx43 antibodies at intercalated disks (Fig. 2C) as well as at mitochondria under normoxic conditions (Fig. 2A) and after ischemic preconditioning (Fig. 2B), whereas no particles were detected in control preparations in which the primary antibody had been omitted (Fig. 2D).

Using immunohistochemistry and confocal laser scan microscopy, a co-localization of Cx43 with succinate-ubiquinol oxidoreductase (Ox-Phos complex II) was detected in cultured mouse atrial HL-1 myocytes (Fig. 3), and purified pig mitochondria stained positive for both Cx43 and cytochrome *c* (Fig. 4A), confirming the localization of Cx43 at mitochondria.

Western blot analysis detected Cx43 in mitochondria isolated from pig myocardium as well as in mitochondria, which were extracted from isolated pig cardiomyocytes. Both preparations were negative for the endothelial marker protein CD31. In the myocardial as well as in the

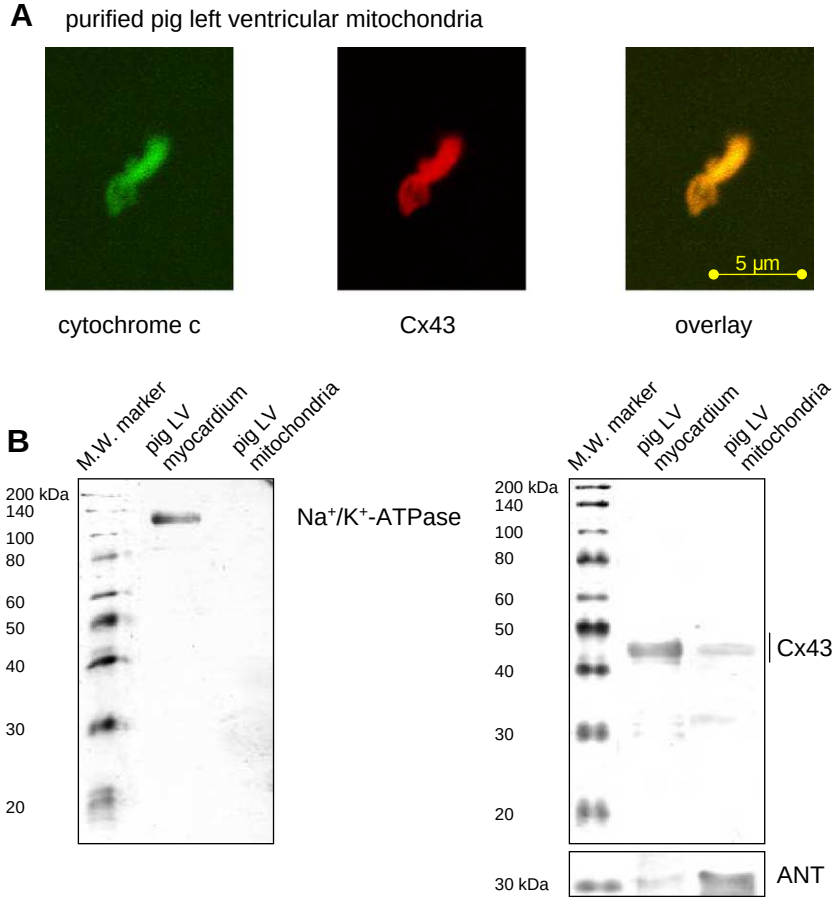

Fig. 4. Cx43 is present in pig left ventricular mitochondria. (A) Purified pig left ventricular mitochondria were stained with antibodies against cytochrome *c* (green) and Cx43 (red) and analyzed by confocal laser scan microscopy. Merged image shows co-localization of pixels in yellow. (B) Western blot analysis was performed for Na^+/K^+-ATPase (left panel) or Cx43 and ANT (right panel) on pig total LV myocardial proteins and on pig LV mitochondrial proteins (30 µg/lane) in order to demonstrate the purity of the mitochondrial preparations as well as the specificity of the antibodies.

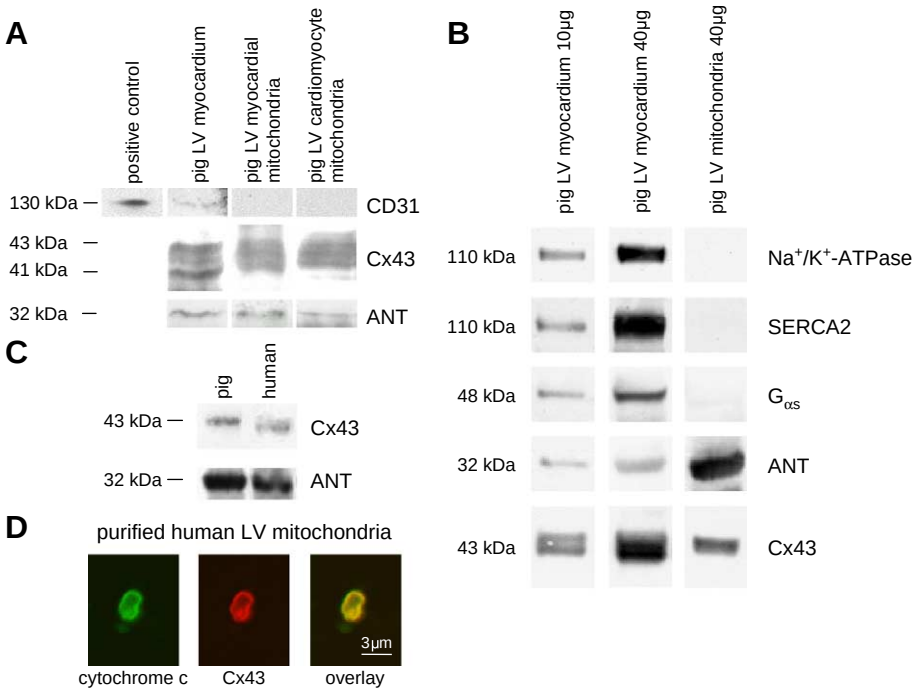

Fig. 5. Cx43 is localized at pig and human left ventricular mitochondria. (A) Western blot analysis was performed for CD31, Cx43 and ANT on total pig left ventricular (LV) myocardium, or on mitochondria, which where extracted from isolated pig LV cardiomyocytes or from pig LV tissue (30 µg/lane). Additionally, a positive control for the CD31 antibody was used. (B) To demonstrate the purity of the mitochondrial extracts, Western blot analysis for Cx43 was performed using either the same protein amount (40 µg) of LV myocardial and mitochondrial proteins or the same Cx43 signal intensities between the control LV myocardial and the mitochondrial proteins, which was achieved by loading 10 µg myocardial proteins and 40 µg mitochondrial proteins. Na^+/K^+-ATPase, $G_{\alpha s}$-protein, SERCA2 and ANT were used as markers of sarcolemma, cytosol, sarcoplasmic reticulum and mitochondria, respectively. (C) Western blot analysis was performed for Cx43 and ANT on purified LV myocardial pig and human mitochondria. (D) Purified human LV myocardial mitochondria were stained with antibodies against cytochrome *c* (green) and Cx43 (red) and were analyzed by confocal laser scan microscopy. Merged image shows co-localization pixels in yellow.

mitochondrial protein extracts, three typical Cx43-specific bands were detected which represent different degrees and/or epitopes of Cx43 phosphorylation (Fig. 5A, for review, see [3]). In all preparations from pig myocardial mitochondria, no immunoreactive signals were detected for markers of the plasma membrane (Na^+/K^+-ATPase, $G_{\alpha s}$ protein), the sarcoplasmic reticulum (SERCA2), and the cytosol ($G_{\alpha s}$) using Western blot analysis (Fig. 5B). In extracts, which stained positive for the inner mitochondrial membrane protein adenine nucleotide transporter (ANT), total Cx43 was present (Fig. 5B). Using Western blot analysis, Cx43 was also detected in mitochondria isolated from hypertrophied human left ventricle (Fig. 5C). Furthermore, purified human LV myocardial mitochondria showed immunoreactivities for both Cx43 and cytochrome *c* as demonstrated by confocal laser scan microscopy (Fig. 5D).

Also rat heart mitochondrial preparations purified by FACS sorting after labeling with MitoTracker Red showed immunoreactivity to Cx43 at a level that represented 16.3±8.5% of that found in intercalated disk fractions while showing no immunoreactive signal for markers of the plasma membrane (Na^+/K^+-ATPase) (Fig. 6A).

To exclude the possibility that the lack of Na^+/K^+-ATPase immunoreactivity in mitochondrial fractions represented a too low concentration of the protein as compared with Cx43, serial dilutions of the intercalated disk fraction from a normoxic rat heart were compared for Na^+/K^+-ATPase and Cx43 signals with those found in the mitochondrial fraction. As seen in Fig. 6B, when comparing similar amounts of Cx43 in the mitochondria and in the diluted intercalated disk fractions (lanes 2 and 7), Na^+/K^+-ATPase immunoreactivity was only detected in the intercalated disk fraction but not in the mitochondrial fraction.

To demonstrate the specificity of the polyclonal anti-Cx43 antibody, Western blot analysis was performed on total protein extracts of ventricular tissues from $Cx43^{fl/fl}$, untreated $Cx43^{Cre\text{-}ER(T)/fl}$ or 4-OHT injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice. Cx43 was present in the ventricles of $Cx43^{fl/fl}$ and to 56.8±8.9% of $Cx43^{fl/fl}$ mice in untreated $Cx43^{Cre\text{-}ER(T)/fl}$ mice. In 4-OHT-injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice, only 3.4±0.9% Cx43 of $Cx43^{fl/fl}$ mice was found. In isolated mitochondria, Cx43 was detected in $Cx43^{fl/fl}$, at low level in untreated $Cx43^{Cre\text{-}ER(T)/fl}$ mice, but not in 4-OHT injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice. Confocal laser scan microscopy demonstrated Cx43 immunoreactivity in mitochondria isolated from $Cx43^{fl/fl}$ mice, to a smaller amount in mitochondria of untreated $Cx43^{Cre\text{-}ER(T)/fl}$, but not in mitochondria of 4-OHT injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice. All mitochondria were positive for the mitochondria marker protein cytochrome *c* (Fig. 7).

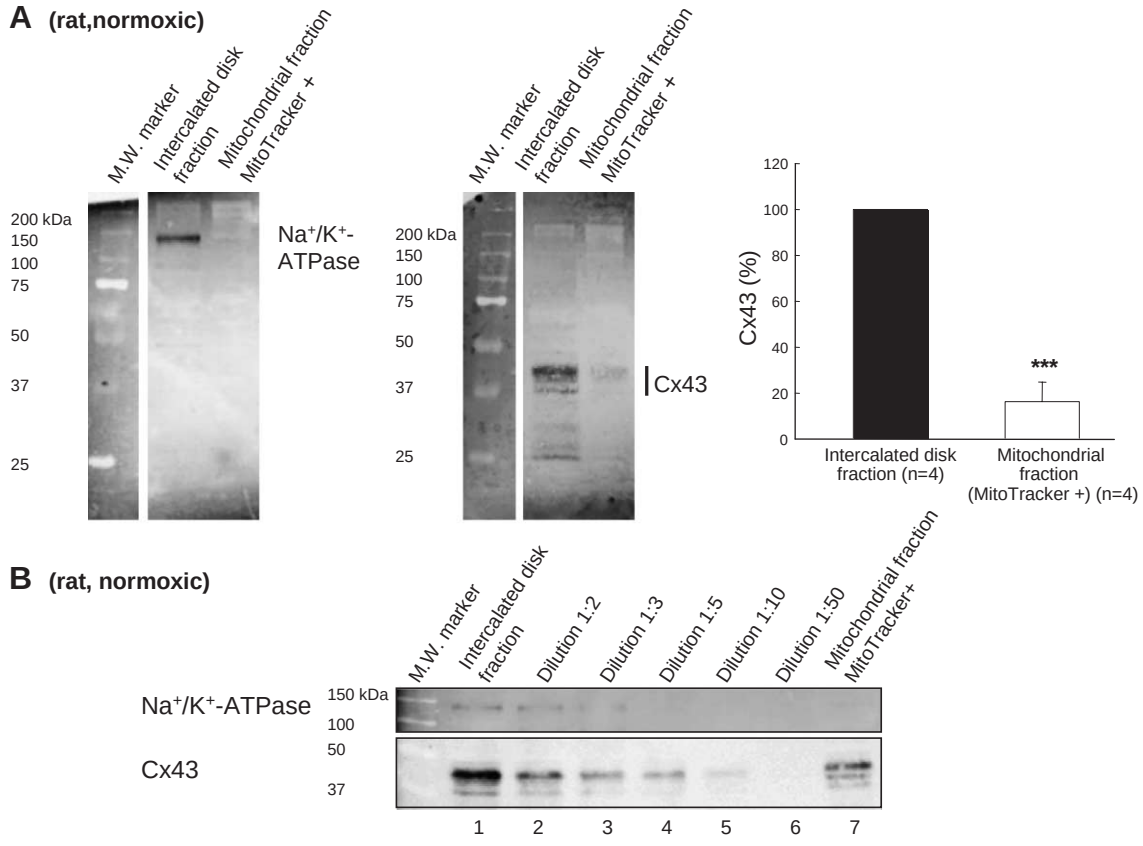

Fig. 6. Cx43 is present in rat myocardial mitochondria. (A) Representative Western blots performed on 10% polyacrylamide gels showing Na^+/K^+-ATPase (left panel) and Cx43 (middle panel) immunoreactivity in intercalated disk and mitochondrial fractions from normoxic rat hearts (15 μg protein/lane). Right panel: Mean mitochondrial Cx43 immunoreactivity (white bar) represented as percentage of that found in intercalated disk fractions (black bar). *** ($p<0.001$) indicates significant differences vs. intercalated disc fraction. (B) Western blots showing Cx43 (71-0700 rabbit antibody) and Na^+/K^+-ATPase immunoreactivity in serial dilutions of the intercalated disk fraction and on the mitochondrial fraction from normoxic rat hearts (10 μg protein/lane).

3.2. IP and mitochondrial Cx43 content

In a rat model of global ischemia, an increased amount of Cx43 at mitochondria following only two cycles of 5 min ischemia/reperfusion was detected by Western blot analysis (262±63% of that found in mitochondria obtained from normoxic hearts, $n=4$, Fig. 8). An enhanced protein level of Cx43 could not be seen in intercalated disk fractions (data not shown). The analysis of the phosphorylation status of Cx43 showed that more than 80% of the Cx43 found in the mitochondria before (93±4%) or following (84±4%, $p=$ns vs. mitochondria from normoxic hearts) the preconditioning cycles was phosphorylated. Only a slight signal was observed with the antibody that detects only non-phosphorylated forms of Cx43 (data not shown).

Analysis with immuno-electron microscopy of myocardial samples from rat hearts showed that the mean number of Cx43-bound colloidal gold spheres per mitochondrion was significantly higher in hearts submitted to IP cycles than in control hearts, which were kept for 20 min under normoxic conditions (Fig. 2A and B, mean values of 1.52±0.17 vs. 0.62±0.13, respectively, $p<0.05$).

In the pig model of low-flow ischemia [20], heart rate, left ventricular pressure, LV dP/dt, coronary arterial pressure, and coronary blood flow did not differ between non-preconditioned and preconditioned hearts throughout the protocol (Table 1). Also, the area at risk (50.8±2.9% vs. 47.4±2.1% of left ventricle) and the subendocardial blood flow at 5 min ischemia (0.04±0.01 ml/min/g vs. 0.05±0.01 ml/min/g) were similar between groups, but infarct size was significantly reduced in preconditioned (6.5±2.5% of area at risk) compared to non-preconditioned hearts (20.1±1.6% of area at risk, $p<0.05$). At the end of 90 min ischemia, there was no difference in the mitochondrial total Cx43 level normalized to ANT between the anterior and posterior wall of non-preconditioned pig hearts (1.1±0.2). However, in preconditioned pig hearts, the ratio of total Cx43 to ANT was 3.4±0.7 fold greater in purified mitochondria extracted from the preconditioned anterior wall than in the control posterior wall (Fig. 9A and B, $p<0.05$). ANT was similarly expressed in mitochondria of ischemic and precondi-

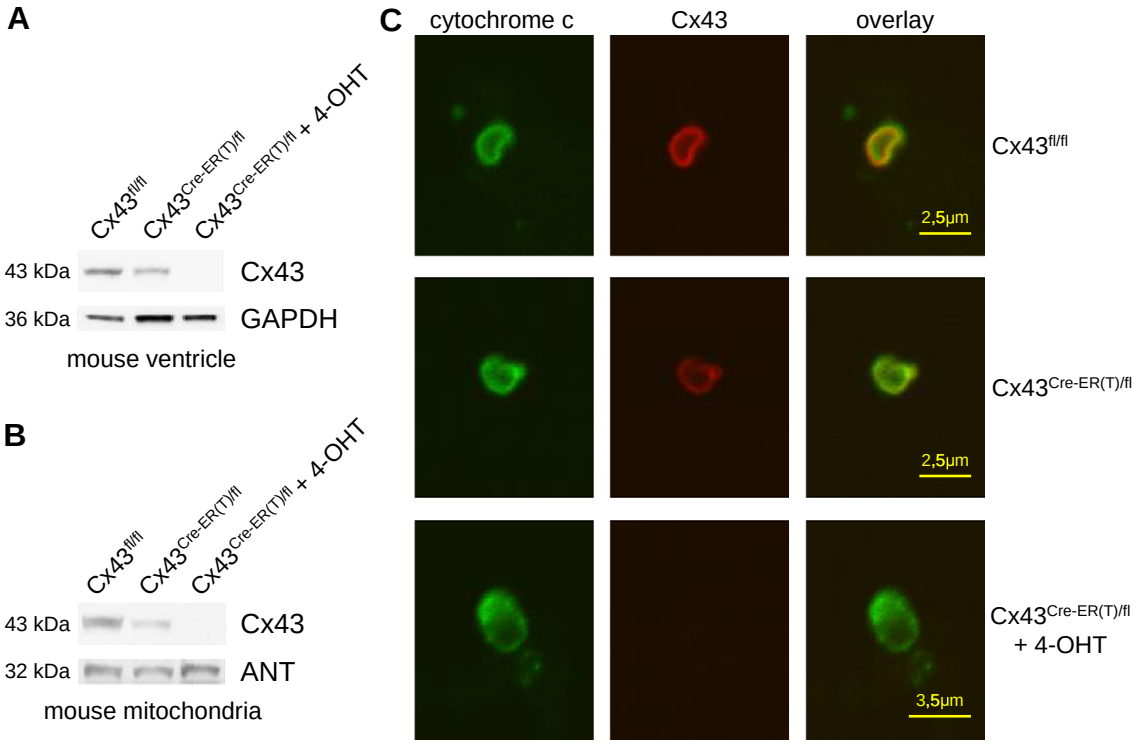

Fig. 7. Specificity of the anti-Cx43 antibody. (A) Western blot analysis was performed for Cx43 (upper panel) or as loading control for GAPDH (lower panel) on 30 µg ventricular protein extracts of $Cx43^{fl/fl}$, untreated $Cx43^{Cre\text{-}ER(T)/fl}$ or 4-hydroxytamoxifen (4-OHT) injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice. (B) Western blot analysis was performed for Cx43 (upper panel) or for the mitochondrial marker protein ANT (lower panel) on 30 µg protein extracts of ventricular mitochondria isolated from $Cx43^{fl/fl}$, untreated $Cx43^{Cre\text{-}ER(T)/fl}$ or 4-hydroxytamoxifen (4-OHT) injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice. (C) Mitochondria isolated from the ventricles of $Cx43^{fl/fl}$, untreated $Cx43^{Cre\text{-}ER(T)/fl}$ or 4-hydroxytamoxifen (4-OHT) injected $Cx43^{Cre\text{-}ER(T)/fl}$ mice were stained with antibodies against Cx43 (red) or the mitochondrial marker cytochrome *c* (green) and analyzed by confocal laser scan microscopy. Merged image shows co-localization pixels in yellow.

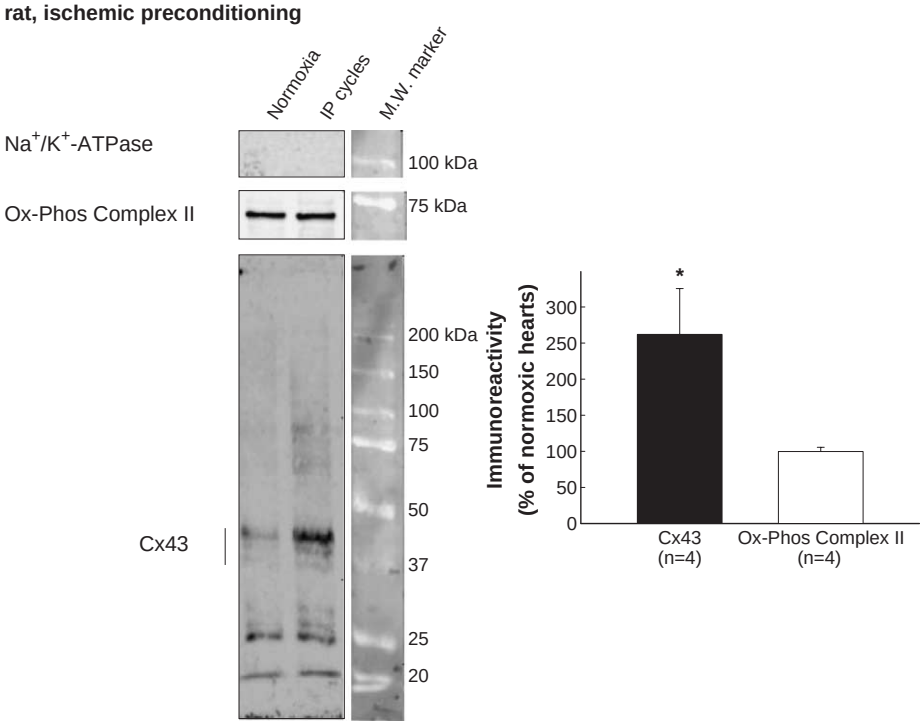

Fig. 8. Cx43 content in rat myocardial mitochondria after ischemic preconditioning. Left panel: Representative Western blots showing immunoreactivity to Cx43, Ox-Phos Complex II and Na^+/K^+-ATPase in mitochondrial fractions from normoxic rat hearts and from hearts submitted to IP cycles (12 µg protein/lane). Right panel: Mean mitochondrial Cx43 (black bars) and Ox-Phos Complex II (white bars) immunoreactivity, as percentage of that found in mitochondrial fractions from normoxic hearts. * ($p<0.05$) indicates significant differences vs. normoxic hearts.

Table 1

		Baseline	5 min ischemia	85 min ischemia
Heart rate (bpm)	Group 1	98±2	99±2	102±3
	Group 2	97±4	98±5	98±4
LV end-diastolic pressure (mm Hg)	Group 1	6.6±1.1	15.1±0.8*	14.3±1.2*
	Group 2	5.5±0.9	13.5±0.7*	13.3±1.6*
LV peak pressure (mm Hg)	Group 1	90±2	78±1*	79±1*
	Group 2	91±3	85±2	82±2*
LV d*P*/d*t*max (mm Hg/s)	Group 1	1383±70	1039±53*	1164±84
	Group 2	1244±110	1013±55	1020±88
Coronary arterial pressure (mm Hg)	Group 1	111±2	27±1*	29±1*
	Group 2	116±4	29±2*	28±2*
Coronary blood flow (ml/min)	Group 1	50.3±8.4	6.0±0.6*	6.0±0.5*
	Group 2	45.6±5.2	6.7±1.3*	6.7±1.3*

Group 1: 90 min ischemia; Group 2: ischemic preconditioning+90 min ischemia; bpm: beats per min.
* $p<0.05$ vs. Baseline.

tioned pig hearts when normalized to protein loading by Ponceau S staining (Fig. 9C).

4. Discussion

The present study demonstrates that Cx43 is localized at cardiomyocyte mitochondria and that the mitochondrial content of Cx43 is enhanced by IP in rat and pig hearts.

Cx43 was found in mitochondria of human umbilical vein endothelial cells [15], but was not detected in mitochondria isolated from human myocardium using a proteomic approach, in which, however, a variety of proteins in the range of 43 kDa were not identified [25]. In the present study, we identified Cx43 in mitochondria of mouse, rat, pig and human myocardium using independent techniques such as FACS, immuno-electron microscopy, confocal microscopy, and Western blot analysis demonstrating that the mitochondrial localization of Cx43 is not unique to endothelial cells.

The Cx43 protein contains no leader sequence targeting the precursor protein to the mitochondria as analyzed with MITOPROT and TargetP [26,27]. However, it is possible that Cx43 is imported to mitochondria via the presequence receptors of the TOM and TIM (translocases of the outer and inner membrane, respectively) protein complexes, which are not necessarily dependent on cleavable presequences [28].

In endothelial cells, cellular stress causes an increased translocation of Cx43 to the mitochondria [15]. Similarly, in the present study in rat hearts the mitochondrial Cx43 content was increased by two short (5 min each) preconditioning cycles of ischemia/reperfusion. At present, we cannot differentiate whether the increased mitochondrial Cx43 content represents an increased import of pre-existing (translocation) or of newly synthesized protein from the cytosol or a decreased degradation of Cx43 at an unchanged rate of Cx43 import. However, given the half-life of Cx43 being close to 90 min, a doubling of the Cx43 content in mitochondria by 2 cycles of 5 min ischemia/reperfusion each (20 min) appears unlikely to be achieved by inhibition of Cx43 degradation only. This implies that increased trafficking of Cx43 is involved in the enhanced mitochondrial Cx43 content. A decreased protein degradation might, however, explain the persistent increase in Cx43 during prolonged ischemia, since Cx43 was still found to be increased following 90 min of ischemia in the pig myocardium. Almost all of the Cx43 at the mitochondrial level–before and after IP–was phosphorylated; however, Cx43 in rat heart mitochondria was completely dephosphorylated after 60 min of ischemia (data not shown), indicating that the phosphorylation of mitochondrial Cx43 is susceptible to ischemia as already shown for sarcolemmal

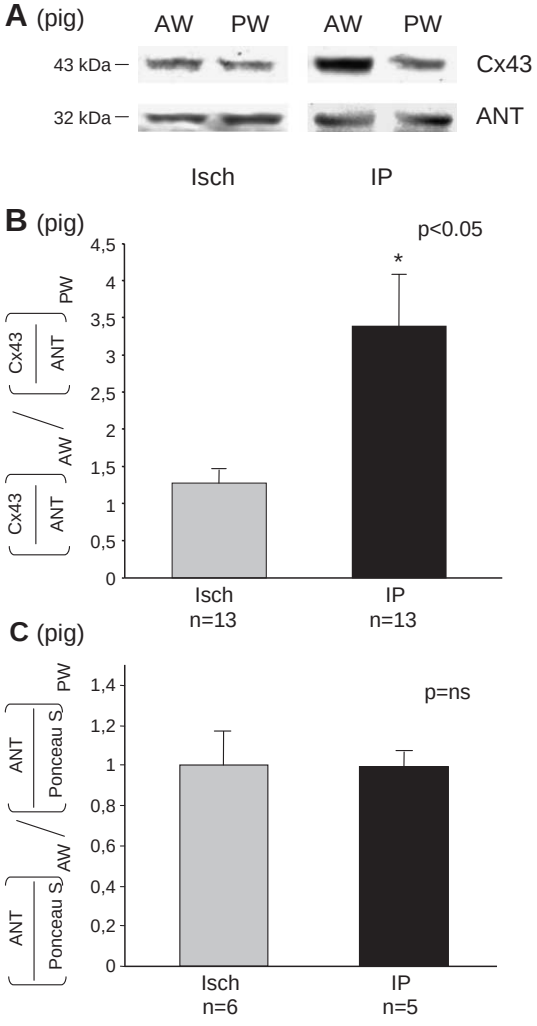

Fig. 9. Cx43 in mitochondria of non-preconditioned and preconditioned pig hearts. (A) Western blot analysis of the Cx43 or ANT protein level in mitochondria isolated from the anterior wall (AW) and posterior wall (PW) of non-preconditioned (Isch) or preconditioned (IP) pig hearts (30 μg/lane). (B) Bar graphs are representing the ratios of the mitochondrial Cx43 level (normalized to ANT) in the AW and PW of pig hearts without or with IP. (C) Bar graphs are representing the ratios of the mitochondrial marker ANT (normalized to Ponceau S staining) in the AW and PW of pig hearts without or with IP.

Cx43 [20,29,30]. The importance of phosphorylation of Cx43 for its translocation to the mitochondria remains to be elucidated in further studies.

The protective effects of Cx43 in IP are independent of cell–cell communication. Experiments in pigs in vivo and in isolated rat hearts revealed a reduction in infarct size, but no effect on ischemia-induced electrical uncoupling by IP [31]. Furthermore, cardiomyocytes isolated from heterozygous Cx43-deficient mice, in contrast to cardiomyocytes from wildtype mice, could not be preconditioned [32]. The involvement of mitochondria in IP has been well described [7]. In the present study we show for the first time that mitochondria of cardiomyocytes contain Cx43. Possibly, the IP-induced increase of mitochondrial Cx43 contributes to the observed cardioprotection by IP. For example, Cx43 could contribute to keep the mitochondrial permeability transition pore–which plays an important role in IP (for review, see [11])–in a closed state, thereby reducing ischemia/reperfusion injury. However, the exact function of mitochondrial Cx43 in the process of IP has to be defined in further studies.

4.1. Clinical implications

While IP is effective in healthy hearts of almost all animal species tested so far [33], there is evidence that it might be no longer operative in hearts post-myocardial infarction or in failing hearts [34,35], both entities associated with a reduction in myocardial Cx43 expression [36,37].

4.2. Methodological aspects

4.2.1. Electron microscopy

The immuno-electron microscopy demonstrated the presence of colloidal-gold-labeled Cx43 antibodies within mitochondria and thereby confirmed the results of the Western blot analysis and confocal laser scan microscopy. The number of particles (1–2 particles per mitochondrion) was low; however, this finding represents the high specificity but low sensitivity of the technique. Due to the distance of about 20 nm between the antigen and the gold-labeled particle as well as the high density of membranes in the mitochondria, the exact localization of Cx43 in mitochondria could not be determined by immuno-electron microscopy. However, since we did not find intercalated disks within 20-nm radius of mitochondrial gold particles, it is unlikely that the Cx43-gold staining corresponds to sarcolemmal gap junctions.

4.2.2. Cx43-antibody

As with each antibody, the sensitivity and specificity of the Cx43 antibody used in the present study can be questioned. However, the identification of three protein bands with molecular weights between 41 and 43 kDa in myocardial and mitochondrial protein preparations of different species as well as the lack of staining in 4-OHT-induced Cx43 knockout mice makes an unspecific, non-Cx43 related cross-reaction of the antibody unlikely. Furthermore, both antibodies directed against total Cx43 (rabbit polyclonal) and non-phosphorylated Cx43 (mouse monoclonal) identified protein bands of around 43 kDa, once more making an unspecific cross-reaction unlikely.

4.2.3. Human tissue

Human tissue was obtained from hypertrophied heart. Since many cardiac diseases are associated with a decrease in sarcolemmal Cx43 [36,37], the level of mitochondrial Cx43 measured in the present study may not be representative for the Cx43 content in normal human tissue.

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