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# Reversible atrial gap junction remodeling during hypoxia/reoxygenation and ischemia: a possible arrhythmogenic substrate for atrial fibrillation

Anna Severino[1*], Maria L. Narducci[1*], Daniela Pedicino[1], Vincenzo Pazzano[1], Ada F. Giglio[1], Luigi M. Biasucci[1], Giovanna Liuzzo[1], Michela Casella[2], Stefano Bartoletti[2], Antonio Dello Russo[2], Gemma Pelargonio[1], Pasquale Santangeli[3], Luigi Di Biase[3], Andrea Natale[3] and Filippo Crea[1]

[1] *Institute of Cardiology, Catholic University of Sacred Heart, Rome, Italy*
[2] *Cardiac Arrhthymia Research Centre, Centro Cardiologico Monzino, Milano, Italy*
[3] *Texas Cardiac Arrhythmia Institute ST David's Medical Center, Austin, USA*

**Abstract.** Alteration of cardiomyocyte gap-junctions and component connexins (Cx) has been suggested to contribute to the development of atrial fibrillation (AF), including postoperative AF. We tested different possible stimuli, such as hypoxia and ischemia, influencing Cx43 and Cx40 expression and distribution in cultured atrial cells (HL-1) and reversibility of these processes after reoxygenation. Western-blot analysis and immunostaining using anti-Cx43, anti-Cx40 and anti-zonula occludens polyclonal antibodies were performed. HL-1 cells exposed to hypoxia for 24 and 48 h showed a reduction of Cx43 protein levels by 75% and 90% respectively ($p < 0.001$). During reoxygenation following 24 h of hypoxia, Cx43 levels increased to reach the basal level within 48 h, while they remained at low level during reoxygenation following 48 h of hypoxia. Furthermore, atrial cardiomyocytes subjected to simulated ischemia (SI) were incubated in normoxic and hypoxic conditions for 3, 6, 9, 12 h. Atrial cardiomyocytes subjected to SI in addition to normoxia showed a progressive reduction of Cx43 levels beginning from 3 h. During SI and hypoxia, atrial Cx43 levels showed an initial decrease after 3 h with a subsequent rescue beginning from 6 h of exposure ($p = 0.001$). Hypoxia and ischemia *per se* downregulate Cx43 protein expression in atrial cardiomyocytes, but protein downregulation is reversible, depending on hypoxia duration and the association of the two triggers. These alterations characterize several conditions and might contribute to the generation of an arrhythmogenic substrate leading to AF onset and/or maintenance.

**Key words:** Atrial fibrillation — Connexins — Ischemia — Reoxygenation — Atrial cardiomyocytes

## Introduction

### *Electrical remodeling, cardiac connexins and atrial fibrillation*

Electrical remodeling plays a key role in the pathophysiology of atrial fibrillation (AF), the most common sustained arrhythmia (Nattel 2002, 2004). In this setting, alterations of cardiac connexins may be looked up as an important mechanism underlying the onset and/or the maintenance of the arrhythmia.

### *Cardiac connexins expression and distribution in atrial fibrillation*

Beauchamp et al. (2006) demonstrated that connexins Cx40 and Cx43 play a key role in the propagation of atrial impulse. Some studies on human atrial samples of patients with chronic AF reported redistribution of Cx43 and Cx40 labeling towards the lateral borders of atrial myocytes and decreased levels of Cx43 *per* myocyte in both right atrial appendage and lateral

Correspondence to: Maria Lucia Narducci, Institute of Cardiology, Catholic University of Sacred Heart, Largo A. Gemelli 8, 00135 Rome, Italy
E-mail: lianarducci@yahoo.it
[*]These authors contributed equally to this work.

free wall (Kostin 2001; Kanagaratnam et al. 2002; Takeuchi et al. 2006), as well as a reduction in Cx40 protein and mRNA levels (Nao et al. 2003; Wilhelm et al. 2006). On the other hand, some studies have reported increased Cx40 levels in patients with chronic AF (Polontchouk et al. 2001; Wetzel et al. 2005); consequently, data on this subject are still conflicting (Chaldoupi et al. 2009).

*Cx40 and Cx43 in postoperative AF*

Among the clinical spectrum of AF, those occurring in the postoperative period in patients undergoing coronary artery bypass grafting (CABG) are associated with a worse outcome (Maisel 2001), but the underlying mechanisms remain unclear. Alterations of gap junctions during bypass surgery, possibly triggered by an ischemic insult, may predispose to the occurrence of post-CABG AF (Yeh et al. 2002). Dupont et al. found increased Cx40 levels in atrial samples of patients with postoperative AF (Dupont et al. 2001), but in a more recent study Cx43 and Cx40 proteins have been found significantly reduced in patients undergoing cardioplegic cardiac arrest during CABG surgery (Li 2009).

*Aim of the study*

While regulation of ventricular connexin has been investigated in several *in vitro* models, atrial connexin have been poorly investigated (Beauchamp et al. 2006). In this study, we evaluated connexin expression and distribution in atrial cardiomyocytes (HL-1 cells) and the effects of hypoxia/reoxygenation, ischemia in association with normoxia and hypoxia, pro-inflammatory stimuli, neurotransmitters and angiotensin II (Ang II) on Cx43 and Cx40 in the same cells. These cells, derived from mouse atrial cardiomyocytes, retain morphological, biochemical, and electrophysiological properties of differentiated atrial cardiomyocytes (Claycomb et al. 1998; White 2004).

## Materials and Methods

*Culture of HL-1 cells*

HL-1 cells were cultured in Claycomb medium (JRH Biosciences) supplemented with 10% FBS (JRH Biosciences), L-glutamine 2 mM, norepinephrine 0.1 mM and penicillin/streptomycin 100 U/ml 100 μg/ml.

*Cell exposure to hypoxia and reoxygenation*

Subconfluent cells were maintained in a humidified incubator in 21% $O_2$ (normoxia) or in a humidified sealed chamber (Billups-Rothenburg, Del Mar, CA) gassed with 3% $O_2$ (hypoxia) for 2, 4, 6, 16, 20, 24 and 48 h. After 24 and 48 h of hypoxia, HL-1 cells were replaced in normoxia conditions for 8, 24, 30 and 48 h (reoxygenation). A total of five experiments were carried out for each time.

*Cell exposure to simulated ischemia*

The cells were subjected to simulated ischemia for 3, 6, 9 and 12 h, by replacing the cell medium with an "ischemia buffer" that contained (in mM): NaCl 118, $NaHCO_3$ 24, $NaH_2PO_4$ 1,

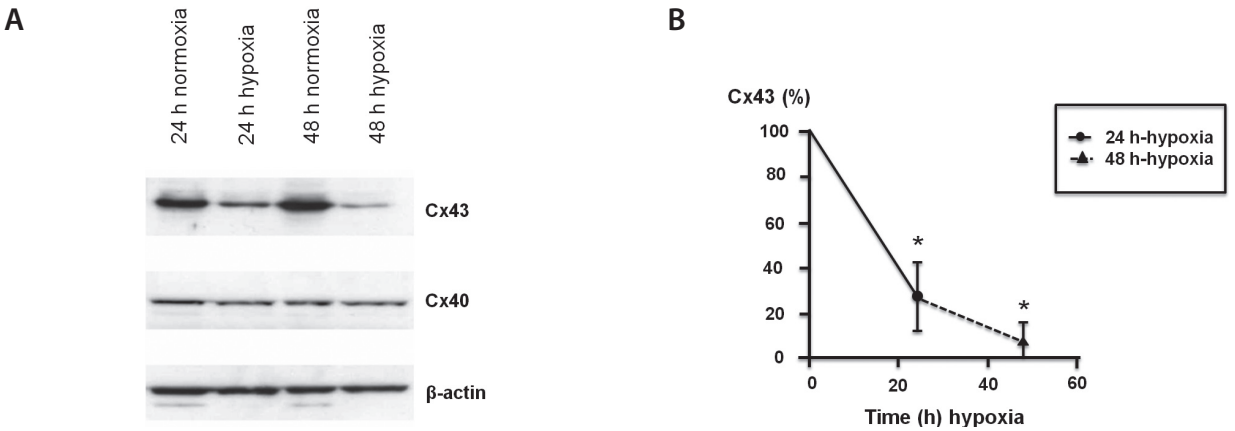


**Figure 1.** Effects of hypoxia on Cx43 and Cx40 protein expression in atrial cells. **A.** Representative blots for the exposure times (24 and 48 h), showing pairs of normoxic and hypoxic samples. Equivalency of loading was verified with an antibody against β-actin. **B.** Quantitative densitometric analysis of Cx43 protein at different time points (0, 24, 48 h of hypoxia). Each value was divided by its corresponding β-actin value. Values were normalized to control (normoxic cultures) values, which were set as 100. * $p < 0.001$, at 24 and 48 h of hypoxia as compared to normoxic cultures. Data are means ± SE; $n = 5$.

$CaCl_2$-$2H_2O$ 2.5, $MgCl_2$ 1.2, sodium lactate 20, KCl 16, 2-deoxyglucose 10 (pH adjusted to 6.2). Contemporaneously, the cells were incubated at 37°C in normoxic (21% $O_2$) and hypoxic (3% $O_2$) conditions during the entire simulated ischemia period.

*Cell incubation with C-reactive protein, cytokines, neurotransmitters and angiotensin II*

HL-1 cells were treated with 1, 5, 10, 20 and 50 ng/ml of human recombinant (hr) C-reactive protein (CRP; Calbiochem, San Diego, CA), with hr interleukins IL-6 and IL-12 (1, 10, 100 ng/ml) (Biodesign, Saco, ME), hr tumor necrosis factor-alpha (TNF-α) (1, 5, 10 ng/ml) (Biodesign, Saco, ME), acetylcholine (Ach) and epinephrine (Epi) (Sigma-Aldrich) (1, 10, 100 μM) or hr angiotensin II (Ang II) (Sigma-Aldrich) (0.01, 0.1, 1 μM) for 24 and 48 h. A total of three experiments were carried out for each time.

*RNA extraction and qRT-PCR*

Total RNA was extracted from HL-1 cells by using Trizol reagent (Invitrogen). 0.5 μg of total RNA was reverse transcribed by iScript cDNA synthesis kit (Biorad). Quantitative polymerase chain reaction (qPCR) was performed by SYBR Green method on an iQ5 Real Time instrument (Biorad). The results were normalized for the β-actin housekeeping gene.

*Cx43 and Cx40 expression*

HL-1 cells were lysed in lysis buffer (in mM): Tris-HCl 50 (pH 7.4), EDTA 5, NaCl 250, NaF 50, 0.1% Triton X-100, $Na_3VO_4$ 0.1, phenylmethylsulfonyl fluoride 1, and 10 μg/ml leupeptin; for 30 min on ice. Lysates were centrifuged at 14,000 × *g* for 10 min at 4°C. Proteins were resolved through 10% acrylamide SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed with anti-Cx40 (C-20), anti-Cx43 (H-150) polyclonal antibodies (Santa Cruz) and anti-β-actin (clone AC-74) monoclonal antibody (Sigma-Aldrich). To detect the phosphorylated forms of Cx43 the following antibodies were used: anti-Cx43 (Tyr 265) and anti-Cx43 (Ser 279/282) polyclonal antibodies (Santa Cruz).

*Cx43 distribution in cardiomyocytes*

HL-1 cells were grown on gelatin/fibronectin-coated coverslips and were fixed with 4% paraformaldehyde in PBS (pH 7.2) for 15 min at room temperature. The fixed cells were permeabilized with PBS/1% TRITON X-100 for 5 min and were immunostained with the following primary antibodies: rabbit polyclonal anti-Cx43 antibodies (Santa Cruz) and anti-zonula occludens (ZO-1) (clone 1A12, Zymed). Incubation with fluorescein-conjugated antibodies was performed. Samples were examined by qualitative immunofluorescence on Olympus confocal laser scanning microscope.

*Statistics*

Data were expressed as mean ± SEM. Normality of data was assessed by Kolmogorov-Smirnov test. Differences were analyzed with the one way-repeated measures ANOVA using Greenhouse-Jeisser post-hoc testing. A value of $p < 0.05$ was

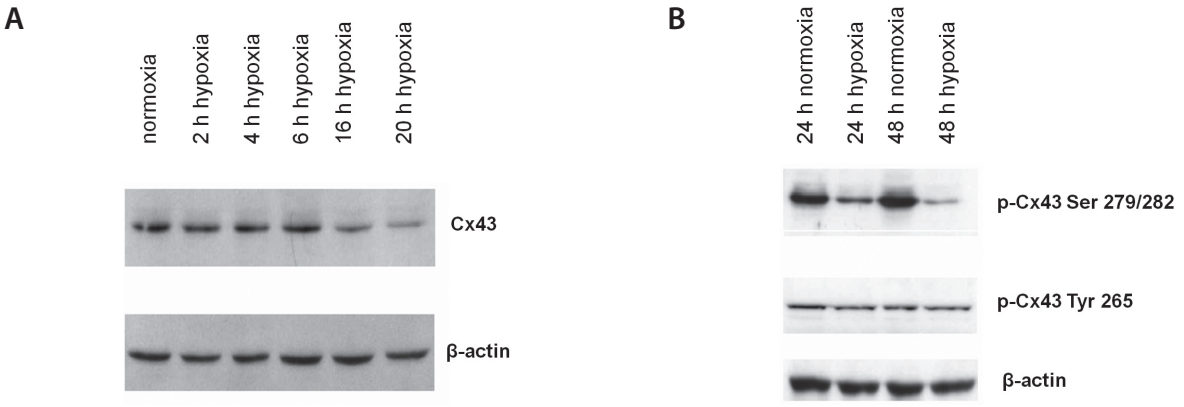


**Figure 2.** Time-dependent effect of hypoxia on total Cx43 protein levels and Cx43 phosphorylated isoforms in atrial cells. **A.** Representative blots for each of the exposure times of 2, 4, 6, 16 and 20 h hypoxia and for normoxia. Equivalency of loading was verified with an antibody against β-actin. Total of three experiments was performed, with similar results. **B.** Effects of 24 and 48 h of hypoxia on phosphorylated isoforms of Cx43. Representative blots for the exposure times (24 and 48 h), showing pairs of normoxic and hypoxic samples of Ser 279/282 and Tyr 265 phosphorylated Cx43 (p-Cx43). Equivalency of loading was verified with an antibody against β-actin (lower panel). Total of 3 experiments was performed, with similar results.

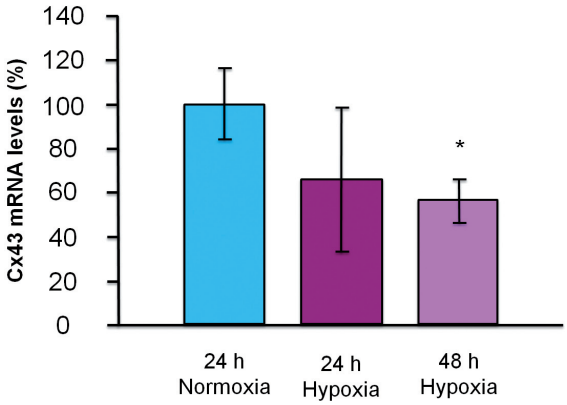


**Figure 3.** The effect of 24 and 48 h of hypoxia on Cx43 mRNA levels in atrial cells. Quantitative analysis of Cx43 mRNA. Atrial cells cultures were exposed to normoxia for 24 h (blue column), to hypoxia for 24 h (purple column) and 48 h (lilac column). Cx43 mRNA was analyzed using RT-PCR. Each value was divided by its corresponding β-actin value. Values are normalized to control values (normoxic cultures), which were set as 100%. * $p = 0.007$, compared to control.

considered significant. Statistical analysis was performed with SPSS software (ver. 15) and graphics were performed using Graph Pad software.

## Results

### *Effects of hypoxia on Cx43 and Cx40*

#### *Cx43 levels*

In Claycomb atrial cells, total Cx43 protein levels at 24 and 48 h of hypoxia decreased to 27 ± 5% ($p < 0.001$) and to 8 ± 4% ($p < 0.001$) of the normoxic level, respectively (Fig. 1A, B). As seen by representative blots (Fig. 2A), the effect of hypoxia on Cx43 protein levels was time-dependent. Beginning from 16 h of hypoxia, there was a significant reduction in total Cx43, protein levels decreased to 50 ± 5% ($p = 0.02$). Of note, during hypoxia the two phosphorylated isoforms of Cx43 (279/282 Ser- and 265 Tyr-phosphorylated Cx43) showed different trends: 279/282 Ser-phosphorylated Cx43 levels at 24 and 48 h of hypoxia decreased to 53 ± 5% ($p = 0.003$) and 20 ± 3% ($p < 0.001$) respectively, while 265 Tyr-phosphorylated Cx43 levels remained permanently unchanged (Fig. 2B). Thus, in atrial cardiomyocytes the reduction of Cx43 protein levels during hypoxia was primarily caused by a reduction of the Ser-phosphorylated isoform. Similarly, Cx43 mRNA levels decreased to 61 ± 0.4% (non significant) at 24 h of hypoxia and to 55 ± 0.1% at 48 h ($p = 0.007$), suggesting that long-term changes in Cx43 protein expression occurred also at a transcriptional level (Fig. 3).

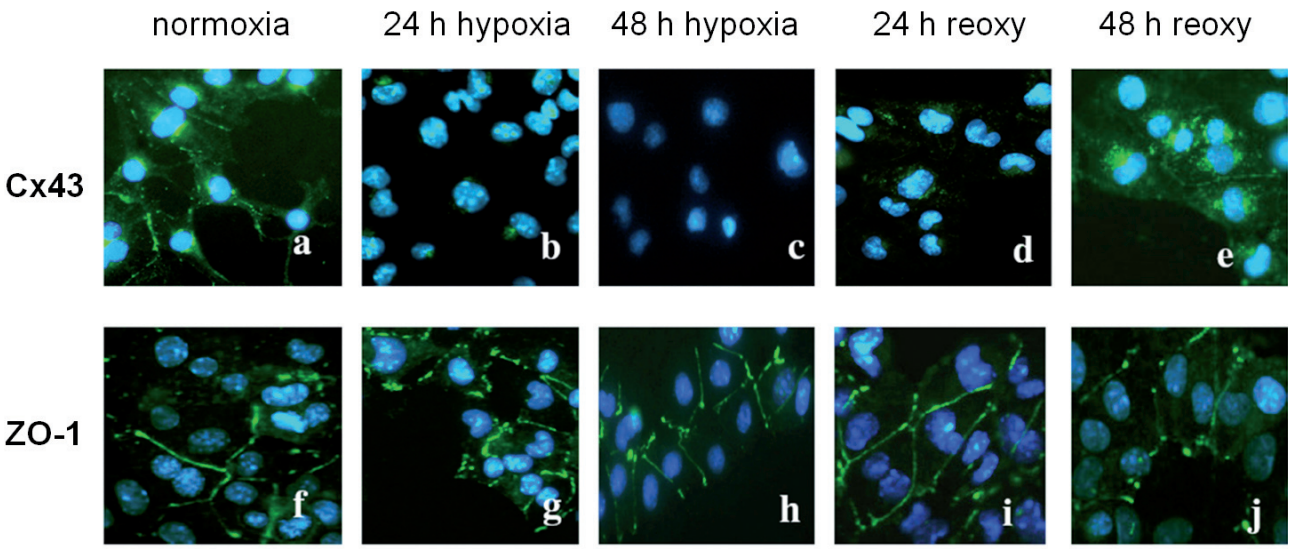


**Figure 4.** Immunofluorescence microscopy of HL-1 cells subjected to normoxia, 24 and 48 h of hypoxia, 24 and 48 h of reoxygenation (reoxy) after 24 h of hypoxia. Immunostaining with polyclonal anti-Cx43 and anti-ZO-1 antibodies was performed (green fluorescence). Immunoreactive signal for Cx43 was concentrated in discrete spots at sites of intercellular apposition and in the cytoplasm (**a**). Immunoreactive signal for ZO-1 was concentrated solely at cell membranes (**f**). After 24 h (**b**) and 48 h (**c**) of hypoxia, green Cx43 staining was dramatically lost, while the intensity of ZO-1 in HL-1 cells was found to be unaltered after hypoxia (**g**, **h**) as compared to normoxic cells (f). Only after 48 h of reoxygenation (**e**), but not after 24 h (**d**), Cx43 immunoreactive signal reached basal levels. The intensity of ZO-1 in HL-1 cells subjected to 24 (**i**) and 48 h (**j**) of reoxygenation was apparently similar to normoxic atrial myocytes. Nuclei were stained blue with DAPI.

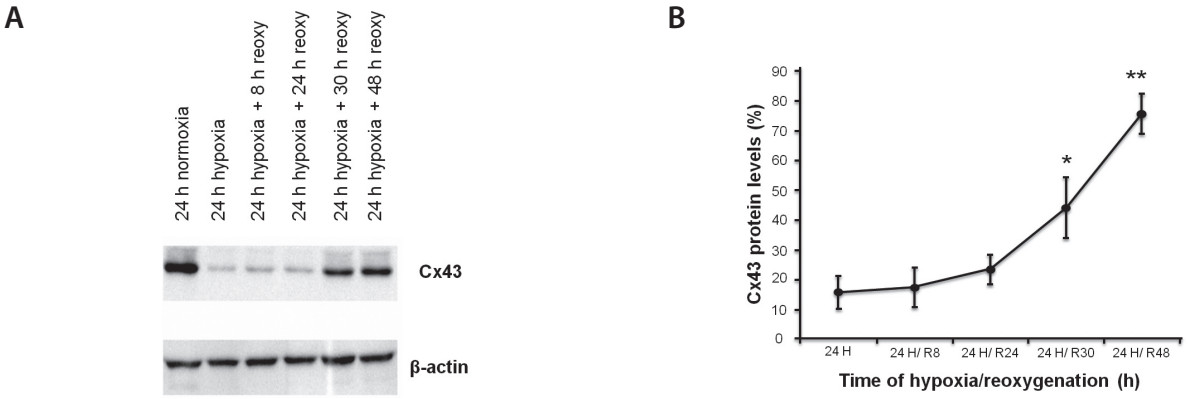


**Figure 5.** Effect of reoxygenation on Cx43 protein levels after 24 h of hypoxia. **A.** After 24 h hypoxia, atrial cells were exposed to 8, 24, 30 and 48 h of reoxygenation (reoxy). Equivalency of loading was verified with an antibody against β-actin. **B.** Quantitative densitometric analysis of Cx43 protein at the time-points considered (24 h hypoxia (H) followed by 8, 24, 30 and 48 h reoxygenation (R). Each value was divided by its corresponding β-actin value. Values were normalized to control (normoxic cultures) values, which were set as 100. * $p = 0.05$, ** $p = 0.001$, compared to 24 h hypoxia. Data are means ± SE; $n = 5$.

*Cx43 distribution*

Figure 4 shows representative images of atrial cells during normoxia and after 24 and 48 h of hypoxia obtained by confocal microscope. In normoxic cells intense Cx43 immunostaining was concentrated in discrete spots on intercellular apposition and in the cytoplasm (Fig. 4a). In contrast, signal for Cx43 was substantially absent in atrial cells subjected to 24 or 48 h of hypoxia (Fig. 4b, c). Of note, ZO-1 was homogeneously distributed on cellular membranes of normoxic atrial myocytes, while it was absent in the cytoplasm (Fig. 4f). The intensity of ZO-1 after 24 or 48 h of hypoxia was found to be unaltered as compared to normoxic atrial cells (Fig. 4g, h).

*Cx40 levels*

Hypoxia did not significantly affect Cx40 protein levels in atrial cells (Fig. 1A).

***Effects of reoxygenation on Cx43 protein levels***

After 24 h of hypoxia, total Cx43 protein levels increased gradually during reoxygenation to reach the basal level within 48 h ($p = 0.001$) (Fig. 5A, B). Accordingly, immunofluorescence analysis for Cx43 in atrial myocytes subjected to 24 h of hypoxia confirmed that Cx43 protein downregulation was reversible after 48 h of reoxygenation (Fig. 4e). In contrast, Cx43 protein levels remained

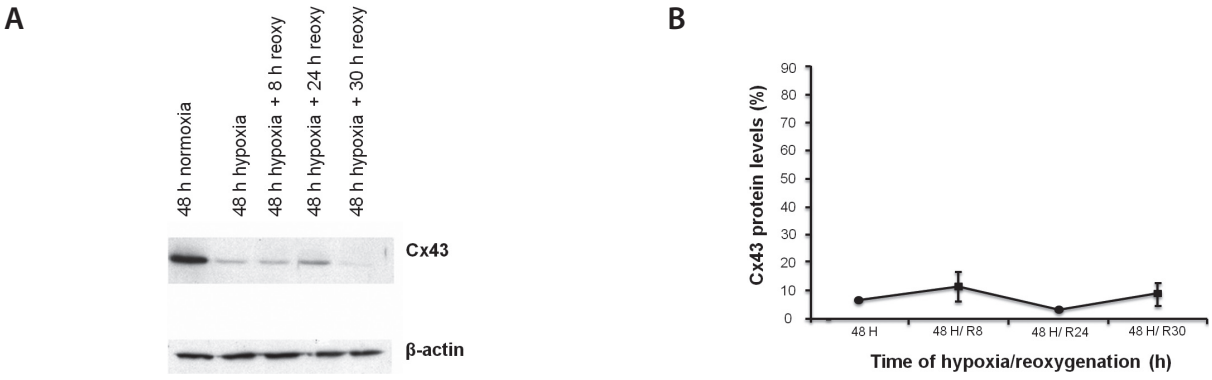


**Figure 6.** Effect of reoxygenation on Cx43 protein levels after 48 h of hypoxia. **A.** After 48 h hypoxia, atrial cells were exposed to 8, 24, and 30 h of reoxygenation (reoxy). 48 h of reoxygenation after 48 h of hypoxia were not included for high atrial cell mortality rate. **B.** Quantitative densitometric analysis of Cx43 protein at the time-points considered (48 h hypoxia (H) followed by 8, 24 and 30 h reoxygenation (R)). Each value was divided by its corresponding β-actin value. Values were normalized to control (normoxic cultures) values, which were set as 100. There was no statistically significant change of Cx43 protein levels in atrial cells exposed to reoxygenation after 48 h of hypoxia. Data are means ± SE; $n = 5$.

unchanged during reoxygenation after 48 h of hypoxia (Fig. 6A, B).

### *Effects of simulated ischemia on Cx43 protein levels*

Atrial cells subjected to simulated ischemia and incubated in normoxic conditions showed a progressive reduction of Cx43 levels (Fig. 7). Particularly, after 3 h of exposure, the quantity of Cx43 protein was decreased to 41 ± 14% to reach a plateau (18 ± 8%) after 6 h of simulated ischemia. Surprisingly, atrial cells subjected to simulated ischemia and incubated in hypoxic condition showed an inverse trend with an initial decrease of Cx43 levels to 58 ± 20% after 3 h of treatment and a subsequent upregulation of Cx43 levels beginning from 6 h (66 ± 29%) up to 12 h (90 ± 39%) of exposure ($p = 0.001$; Fig. 7).

### *Effects of cell incubation with CRP, cytokines, neurotransmitters and Ang II*

Incubation with increasing concentrations of CRP, IL-6, IL-12 and TNFα did not affect Cx43 and Cx40 protein levels. Similarly, incubation with increasing concentrations of Ach and Epi or Ang II for 24 and 48 h did not alter Cx43 and Cx40 protein levels.

## Discussion

### *Hypoxia/reoxygenation and connexin remodeling: Cx43 modulation*

Hypoxic-ischemic injury is one of the most studied triggers of connexin remodeling, which in turn is a possible contributing factor to the occurrence of AF (Kanagaratnam et al. 2002). While previous studies assessed connexin modulation only in ventricular myocytes (Beardslee et al. 2000; Kostin 2001; Zeevi-Levin et al. 2005; Matsuschita et al. 2006), this is the first study on connexin remodeling in adult atrial cells, showing that both hypoxia and ischemia are potent modulator of Cx43 redistribution and that the latter can be, in some cases, reversible. In the first part of our study, we observed a significant downregulation of Cx43 expression in atrial cells during hypoxia, confirmed by Western Blot analysis and qualitative immunofluorescence, demonstrating at the same time how the hypoxic insult acts specifically on Cx43, since qualitative immunofluorescence did not confirm the same trend for ZO-1, an essential constituent of intercalated disk required for the proper formation of Cx43 gap junctions (Laing et al. 2007; Palatinus et al. 2011), and for Cx40 protein levels. Moreover, we found that Cx43 protein downregulation in atrial cells during hypoxia is time-dependent, associated with a parallel Cx43 mRNA decrease. Finally, Cx43 protein level reduction after 24 h of hypoxia reversed to almost the pre-hypoxic value during reoxygenation, while the reduction after 48 h of hypoxia was irreversible. The loss of reversibility of Cx43 downregulation after 48 h of hypoxia followed by reoxygenation was probably due to a high apoptotic rate of HL-1 atrial cells induced by long-term hypoxia. The aforementioned data can be partially confirmed by several studies in ventricular myocytes. Zeevi-Levin et al. (2005) demonstrated that 5 h of hypoxia decreased Cx43 protein in rat ventricular myocytes, without a concurrent reduction of mRNA levels. In our study, Cx43 mRNA downregulation at 48 h of hypoxia in atrial cells could be explained by specific regulation of connexins in different species (rat *versus* mouse) and tissues (ventricle *versus* atrium) (Kostin 2001). In keeping with our data in atrial cells, ZO-1 ventricular sarcolemmal expression did not change during hypoxic condition and hypoxia-induced effects on ventricular Cx43

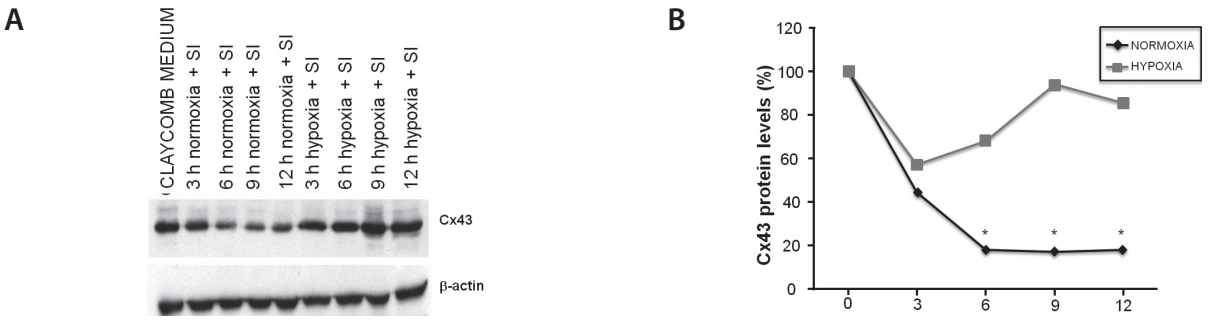


**Figure 7.** Cx43 protein expression during simulated ischemia (SI). **A.** blots for the exposure times of 3, 6, 9 and 12 h for samples incubated respectively in normoxic and hypoxic conditions. Equivalency of loading was verified with an antibody against β-actin. **B.** Quantitative densitometric analysis of Cx43 protein in HL-1 cells cultured in the same experimental conditions as in A. After 6 h of normoxia, total Cx43 protein level was significantly reduced in HL-1 cells treated with SI ($p = 0.001$), but Cx43 protein level did not change during hypoxia + SI treatment. In addition to SI, hypoxia could inhibit the downregulation of Cx43 protein and could be considered as a cytoprotective mechanism induced by oxidative stress. * $p = 0.001$; $n = 5$.

protein level were reversed by reoxygenation (Matsuschita et al. 2006). Moreover, as previous studies in ischemic ventricular myocytes demonstrated (Beardslee et al. 2000; Matsuschita et al. 2006), in our study atrial cardiomyocytes exhibited a progressive reduction of Ser-phosphorylated Cx43. Of note, the loss of serine phopshorylation and the gain of tyrosine phosphorylation have been associated with loss of cell-cell coupling (Lau et al. 1996; Zhou 1999; Barker 2002; Tepass 2002; Kieken et al. 2009). At this regard, our study could be a further confirmation of atrial intercellular uncoupling arising from hypoxic insults, but studies on functional gap junction coupling will be needed to confirm this hypothesis.

*Role of hypoxia and/or ischemia in Cx43 regulation*

In order to further clarify the role of hypoxic-ischemic insult on gap junction activity, we tested the effects of ischemia associated with normoxia and hypoxia on atrial cells. Ischemia is indeed a complex condition involving not only hypoxia, but also a build-up of toxic metabolites, acidosis and energy substrates depletion. This pathophysiological setting, characterized not only by oxygen deprivation but also by metabolic stress, occurs in several cardiac pathological conditions, and could also be considered as an *in vitro* replication of cardioplegic arrest during cardiac surgery (Anselmi et al. 2004). In our study, atrial cells subjected to ischemia and incubated in normoxic conditions showed Cx43 downregulation as atrial cells exposed only to hypoxia. In contrast to this trend, atrial cells subjected to ischemic and hypoxic insult showed an initial decrease of Cx43 levels with subsequent rescue of protein levels beginning from 6 h of exposure. These data highlight the time dependence of Cx43 modulation during the association of hypoxic and ischemic insult. Although this different trend needs to be proved in electrophysiological studies assessing Cx43 hypoxia/ischemia-induced modulation, several studies on neuronal activity provided a functional explanation of an increased Cx43 expression during hypoxia/ischemia (Nakase 2003, 2006; Jaderstad 2010; Orellana et al. 2010). Lin et al. (1998) demonstrated that astrocytes subjected to ischemic stimuli can increase the density and the number of sarcolemmal gap junctions with consequent spreading of toxic metabolities to the neighboring unaffected cells. The different responses to hypoxic and/or ischemic stimuli by atrial cells are strongly dependent by the quality of the insult. We could hypothesize that in the first hours of ischemic stress, the downregulation of Cx43 protein may represent the consequence of hypoxic state (Schulz et al. 2007; Srisakuldee et al. 2009; Mühlfeld et al. 2010). A persistent ischemic stress could upregulate Cx43, promoting the spreading of the ischemic insult in neighboring unaffected cells (Andrade-Rozental et al. 2000; Frantseva et al. 2002a,b; Cusato et al. 2003; de Pina-Benabou et al. 2005; Talhouk et al. 2008).

*Myocardial hypoxia/ischemia and atrial fibrillation*

Several studies have shown the role of myocardial ischemia in the pathogenesis of AF. It has been proved that atrial ischemia promotes AF in dogs (Sinno et al. 2003). In patients with lone AF, Skalidis et al. (2008) demonstrated isolated atrial microvascular perfusion abnormalities. Moreover, it has been recently demonstrated that coronary disease involving the atrial branches (e.g. right coronary atrial branch and left circumflex atrial branch) is an independent predictor for the development of AF early after an acute myocardial infarction, even after adjustment for age, gender, left ventricular ejection fraction and filling pressure, left atrium size, time to reperfusion and TIMI flow after percutaneous revascularization (Alasady et al. 2011). Ahlsson et al. showed a significant correlation between postoperative AF and ischemic myocardial injury assessed by CK-MB (creatin kinase isoenzyme MB) values (Ahlsson et al. 2007). Accordingly, in a prospective observational study, postoperative AF was associated with a lower rate of preoperative therapy with beta-blockers, possibly because of their anti-ischemic effect (Mathew et al. 2004). Particularly, during cardiac surgery with cardiopulmonary bypass and cardioplegic arrest, downregulation of atrial gap junctions (Cx43 and Cx40) occurred (Yeh et al. 2002; Li 2009), while in another study higher levels of Cx40 expression were found in patients that subsequently developed AF (Dupont et al. 2001).

Cx43 is well expressed in atrial myocardial tissue (Vozzi et al. 1999). Several studies on Cx43 expression in human and animal models of AF yielded conflicting results, demonstrating both increased and decreased Cx43 levels (Elvan et al. 1997; Kostin et al. 2002). Similarly, studies on Cx40 expression in AF have also obtained controversial data (Dupont et al. 2001; Kostin et al. 2002; Chaldoupi et al. 2009). The reasons underlying these differences are probably multiple, including study on various animal models and patients with AF of different aetiology, analysis of different myocardial regions (e.g. atrial *versus* ventricular tissue, left atrium *versus* right atrium), and lack of distinction between gap junction alterations inducing AF or provoked by the arrhythmia itself. Concerning the results of our study, we could hypothesize that the stimuli we used may act differently on connexin expression, modulating Cx43 while Cx40 could be influenced by other conditions not tested in the present study. A recent study on animals suggest that downregulation of Cx43 is of major importance in determining myocardial susceptibility to AF development (Bikou et al. 2011), although this finding needs further validation in humans.

*Inflammatory stimuli and connexin expression*

As inflammation of atrial tissue has been documented in patients with AF (Boos 2006), we studied also the effect of pro-inflammatory cytokines and CRP as potential regulators of connexin expression in atrial cells. We failed to find changes in Cx43 and Cx40 protein expression after incubation with increasing concentrations of CRP, IL-6, IL-12 and TNF alpha. Cx43 and Cx40 protein levels did not change either after incubation with Ach, Epi or Ang II. In the particular setting of postoperative AF, the possible relation between this arrhythmia and CRP levels has been studied with conflicting results (Amar et al. 2005; Fontes et al. 2005; Lo et al. 2005; Hogue et al. 2006) and recent evidence suggests no correlation between preoperative and postoperative CRP concentrations and postoperative AF, while a significant correlation was found between ischemic myocardial injury assessed by postoperative CK-MB values (Ahlsson et al. 2007).

*Effect of Ang II on atrial connexin expression*

Current clinical evidence suggests a role of renin-angiotensin system blockade in the prevention of AF (Jibrini 2008). In this study, we failed to find changes in atrial connexin expression after treatment with Ang II, while, in ventricular myocytes, Ang II increased Cx43 expression level (Polontchouk et al. 2002). Thus, the therapeutic effects of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers on AF could be mediated by molecular pathways not involving connexin expression.

## Conclusion

Alterations of intercellular communication through gap junctional connections are likely to play an important role in the pathogenesis of AF. We used a cell model of differentiated atrial cardiomyocytes to investigate the role of potential modulators of connexin remodeling. We found that hypoxia and ischemia are potent modulators of Cx43 expression at both translational and transcriptional levels, without any detectable effect on Cx40 protein levels. Thus, atrial Cx43 downregulation might be independently induced by hypoxia and ischemia and might be reversible after reoxygenation. Prolonged hypoxic-ischemic insult upregulate Cx43 protein levels with a rescue to baseline levels. These different responses to hypoxia and ischemia by atrial Cx43 could be partially responsible of gap junction alterations observed in AF.

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