UNIVERSITAT AUTÒNOMA DE BARCELONA
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DOCTORAL THESIS

ROLE OF CONNEXIN 43 IN ISCHEMIA-REPERFUSION INJURY: EFFECT OF GENETIC CONNEXIN 43 MANIPULATION ON MYOCARDIAL CELL DEATH AND ARRHYTHMIAS

Report presented by Jose Antonio Sánchez García to apply for the degree of Doctor by the "Universitat Autònoma de Barcelona".

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A Núria, Arnau,
mis padres
y Carlos…

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In this part of the thesis I will address to the corresponding persons in the language I speak with them.

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Contents

Abbreviations

4-OHT	4-Hydroxytamoxifen
ACh	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ANT	Adenine nucleotide translocase
BCL	Basic cycle length
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CsA	Cyclosporine-A
Cx32	Connexin 32
Cx43	Connexin 43
Cx43$^{-/-}$	Cx43 knock-out mice
Cx43$^{+/-}$	Heterozygous Cx43 knock-out mice
Cx43KI32	Transgenic knock-in mice
Cx43$^{Cre\text{-}ER(T)/fl}$	Conditional knock-out transgenic model
Cx43$^{Cre/fl}$	Conditional knock-out genotype
Cx43$^{fl/fl}$	Genetic controls from conditional knock-out mice
CyP-D	Cyclophilin-D
dNTPs	Deoxynucleotide triphosphate mix
dV/dt_{max}	Maximal rate of rise of action potential upstroke
Dzx	Diazoxide
ECG	Electrocardiogram
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
ERK-1/2	Extracellular signal-regulated protein - 1/2
ER(T)	Human estrogen receptor

GC	Guanylyl cyclase
GJ	Gap junctions
GPCR	G protein-coupled receptors
GSK3β	Glycogen synthase kinase 3β
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HET	Heterozygous
HOM	Homozygous
Hsp-90	Heat shock protein -90
IP3	Inositol 1,2,5-triphosphate
IPC	Ischemic preconditioning
JAK	Janus kinase
LDH	Lactate dehydrogenase
LVEDP	Left ventricular end-diastolic pressure
LVdevP	Left ventricular developed pressure
LVP	Left ventricular pressure
MANOVA	Repeated-measures analysis of variance
MAPK	Mitogen activated protein kinases
mitoK$_{ATP}$	Mitochondrial ATP sensitive potassium channel
MOPS	3-Morpholinopropane-1-sulfonic acid
MPTP	Mitochondrial permeability transition pore
NAD$^+$	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NBC	Na^+/HCO_3^- co-transporter
NCE	Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
Oxphos CII	Succinate-ubiquinol oxidoreductase
PABA	Para-aminobenzoic acid
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PCR	Polimerase chain reaction

PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMSF	Phenylmethanesulfonyl fluoride
PP	Perfusion pressure
PVBs	Premature ventricular beat
RISK	Reperfusion injury salvage kinases
ROS	Reactive oxygen species
RT	Room temperature
SAFE	Survivor activating factor enhancement
SDS	Sodium dodecyl sulphate
SE	Standard error
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
STAT3	Signal transducer and activator of transcription-3
TBS-T	Tris base, NaCl, Tween 20
TCA	Tricarboxylic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TIM	Translocase of the inner mitochondrial membrane
TOM	Translocase of the outer mitochondrial membrane
TSP	3-(Trimethylsilyl)propionic acid-d4 sodium salt
TTC	2,3,5-triphenyltetrazolium chloride
Txnip	Thioredoxin-interacting protein
VDAC	Voltage-dependent anion channel
VF	Ventricular fibrillation
Vj	Transjuctional voltage
Vm	Transmembrane potential
VT	Ventricular tachycardia
WT	Wild type
ZO-1	Zonula occludens -1

Summary

Ischemia-reperfusion injury contributes to morbidity and mortality in a wide range of pathologies, including ischemic heart disease, the main cardiovascular disease, responsible of a high number of deaths worldwide every year. The most critical determinant of prognosis in patients with acute myocardial infarction is infarct size, which, in turn, correlates with the size of the area at risk and ischemia duration. Nowadays, rapid coronary flow restoration (i.e., reperfusion), either through thrombolysis or primary percutaneous interventions, is the treatment of choice in these patients in order to prevent cardiomyocyte death and improve patient's survival and prognosis. However, and despite the correct application of these therapies, ischemic heart disease continues being a leading cause of mortality and morbidity in the whole world population. This is in part due to the fact that reperfusion causes an additional injury, through mechanisms triggered by flow restoration, that can be prevented, at least in part and in the experimental setting, by drugs applied at the time of reperfusion. This fact has led to an intense research in order to identify therapeutic strategies able to limit myocardial infarction when applied at the time of reperfusion.

One of the candidate targets is Cx43, and the channels that it forms (i.e, GJ channels). Cx43 is the main protein forming GJ in the ventricular myocardium, where it plays an essential role in electrical and chemical coupling between cardiomyocytes. However, previous studies have suggested that these channels play also a role in spreading of cell death during reperfusion, and that their blockade during the first minutes of flow restoration could reduce infarction. Moreover, Cx43 has been also suggested to be involved in endogenous cardioprotective signaling during IPC. However, most of these studies have been conducted using GJ uncouplers having low specificity and lots of side effects. This made necessary to find new strategies to study the role of Cx43 in cell death during myocardial infarction and cardioprotection, including the use of transgenic mice models. Thus, the aims of this thesis were to assess the effects of Cx43 deficiency in transgenic models on cardiac energetic metabolism, tolerance to ischemia-reperfusion injury, susceptibility to ischemic and pharmacological preconditioning, and on the incidence of ventricular arrhythmias.

In our first approach, we used a knock-in mice model, in which Cx43 is replaced by Cx32, a connexin with lower conductivity and permeability. We found that substitution of Cx43 by Cx32 alters myocardial energetic metabolism, with hearts from these mice having reduced ATP levels as compared with hearts from WT animals. Furthermore, isolated, Langendorff-perfused, hearts from HOM Cx43KI32 mice

depicted an increased tolerance to ischemia-reperfusion injury, as denoted by a reduction in infarct size and in LDH release. This effect can be, in part, independent of GJ communication, as isolated cardiomyocytes from these animals also showed some degree of protection. Moreover, IPC was abolished in HOM Cx43KI32 mice, whereas pharmacological preconditioning with Dzx was abolished in both HET and HOM mutant mice.

To assess whether our previous findings were due to the presence of Cx32, we used a conditional knock-out model of Cx43 deficiency ($Cx43^{Cre\text{-}ER(T)/fl}$). In this model, hearts from $Cx43^{Cre/fl}$ animals express, under normal conditions, half of the Cx43 content seen in their corresponding genetic controls ($Cx43^{fl/fl}$). However, 14 days after 4-OHT treatment, hearts from these animals have only a residual Cx43 content, lower than 5%. Using this model we have been able to demonstrate in isolated, Langendorff-perfused, hearts, that Cx43 deficiency is associated with an altered energetic metabolism, an increased resistance to ischemia-reperfusion injury, and an altered susceptibility to preconditioning protection, especially to Dzx. Although the increased resistance to ischemia-reperfusion injury seems to be more dependent on gap junctional communication than in hearts from HOM Cx43KI32 animals, these data confirmed the importance of Cx43 in these effects. Furthermore, we have demonstrated that these effects were independent of a differential activation of cytosolic signaling cascades, including the RISK and SAFE pathways, which is suggestive that activation of these pathways during IPC is upstream of Cx43 deficiency.

Finally, we have demonstrated that both models of Cx43 deficiency, mimicking a reduction in unitary GJ conductance and in the number of GJ channels, respectively, are associated with an increased incidence of both spontaneous and induced ventricular tachyarrhythmias. This finding indicates that the possibility of translation of these therapeutic strategies to the clinical arena requires that they could be applied locally at the area at risk.

In conclusion, this thesis demonstrates an important, and previoulsy unknown, specific role of Cx43 in myocardial energetic metabolism, tolerance to ischemia-reperfusion injury and in endogenous cardioprotection. GJ and Cx43 can become interesting pharmacological targets to improve the clinical outcome in patients with ischemic heart disease. However, translation should wait until the proarrhythmic effects of these treatments are solved.

Introduction

Cardiovascular diseases are the main cause of death and disability in industrialized countries, accounting for more than six millions deaths in 1990, and its prevalence is continuously growing up across the world (Murray and Lopez, 1997). The group of circulatory system diseases was the first cause of mortality in Spain in 2000, with 124,610 deaths (Boix et al., 2004). Ischemic heart disease is one of the main cardiovascular diseases with 39,000 deaths during that year.

The short- and long-term prognosis after an episode of myocardial ischemia, including the incidence of events such as death, malignant arrhythmias, or development of heart failure, correlates well with the extent of myocardial necrosis or infarction (Thompson et al., 1979). In turn, the extension of cell death secondary to a coronary occlusion depends on the area of myocardium submitted to ischemia (area at risk), the presence of residual blood flow and the duration of ischemia (Reimer et al., 1977; Garcia-Dorado et al., 1987). Rapid coronary flow restoration, or reperfusion, either through thrombolysis or primary percutaneous interventions, is the treatment of choice in patients with acute coronary syndrome with ST segment elevation in order to prevent ischemic cardiomyocyte death and improve patient's survival and prognosis (Garcia-Dorado, 2004).

However, reperfusion is associated with additional injury that limits myocardial salvage. It has been demonstrated that when reperfusion is performed in the period of time in which part of the myocardium can be saved, cell death occurs mainly during the first minutes of blood flow restoration, as necrosis, and caused by mechanisms triggered during reperfusion (Piper et al., 1998). This phenomenon, known as reperfusion injury, can be reduced by a number of therapeutic interventions applied at the time of reperfusion (Piper et al., 2004), that have been proved to be successful under experimental conditions. Although the molecular and cellular mechanisms of cell death during reperfusion are not completely understood (Garcia-Dorado et al., 2009), development of effective and clinically useful treatments to prevent reperfusion injury is essential to reduce the impact of ischemic heart disease in our society. Thus, further research effort should be done to find new molecular mechanisms and targets involved in reperfusion injury (Rodriguez-Sinovas et al., 2007).

Connexin 43 (Cx43) is a transmembrane protein expressed in ventricular myocardium and many other tissues. Six molecules of Cx43 leaving a central pore form a hemichannel. The interaction between the extracellular domains of two hemichannels allows the formation of an intercellular channel, which tend to aggregate forming

plaques, known as gap junctions (GJ) (van Veen et al., 2001). GJ are essential for cardiac function, as they mediate intercellular coupling, and thus, synchronized cardiac contraction (Jalife et al., 1999). Interestingly, they have been suggested to play a role in spreading of cell death during reperfusion, as administration of GJ uncouplers at the onset of reperfusion was able to reduce myocardial infarct size in pigs and rats (Garcia-Dorado et al., 1997; Rodriguez-Sinovas et al., 2004). Furthermore, Cx43 has been suggested to be involved in protection by ischemic preconditioning (IPC) (Schwanke et al., 2002). However, the low specificity of tested GJ uncouplers made necessary to develop new strategies to study the role of Cx43 in cell death during myocardial infarction, and in cardioprotection. These new strategies include the use of transgenic models. This thesis tries to clarify the role played by Cx43 on the mechanisms involved in myocardial ischemia-reperfusion injury and protection using two transgenic mice models of Cx43 deficiency. Moreover, the effects of such reductions in GJ intercellular communication on ventricular arrhythmogenesis must be considered before translating any possible treatment into the clinical arena.

1. Consequences of myocardial ischemia-reperfusion injury

Ischemia-reperfusion injury can cause cell death mainly depending on the duration of the ischemic insult. Dead cardiomyocytes are replaced by fibrotic tissue, leading to development of the infarct scar. Depending on scar extension, myocardial remodeling may lead to the appearance of heart failure (Gajarsa and Kloner, 2011). On the contrary, viable cardiomyocytes salvaged by reperfusion, show transient dysfunction, a phenomenon known as myocardial stunning (Braunwald and Kloner, 1982; Heyndrickx, 2006). In addition, both ischemia and reperfusion are associated with a high incidence of ventricular arrhythmias, having different mechanisms depending on the time of appearance.

1.1- Heart failure

Heart failure is a clinical syndrome caused by the inability of the heart to supply sufficient blood flow to satisfy the needs of the body. As commented before, the most important consequence of myocardial ischemia-reperfusion is cell death. After myocardial infarction, compensatory changes in heart geometry and function develop, in a process known as cardiac remodeling (Sabbah and Goldstein, 1993). However, adverse remodeling, with progressive left ventricular dilation and hypertrophy, is associated with the appearance of heart failure, arrhythmogenesis and poor prognosis (Gaudron et al., 1993).

The complete mechanisms leading to cardiac remodeling remain unclear, although it is thought that molecular changes may alter the expression of different proteins. At the cellular level, they may cause myocyte hypertrophy, necrosis, apoptosis, fibrosis, increase fibrillar collagen deposition, and induce fibroblast proliferation (Cohn et al., 2000). These cellular alterations are responsible for wall thinning, ventricular dilation and hypertrophy, observed in patients after myocardial infarction (Anversa et al., 1991). Ventricular dilation increases wall stress in the infarcted ventricle. Moreover, the progression of eccentric hypertrophy and wall thinning induces an increase in left ventricular cavity dimensions at a faster rate than that of myocardial mass. As a consequence, ventricular wall stress is further enhanced, sub-endocardial perfusion is decreased and, eventually, left ventricular ejection fraction is reduced (Gajarsa and Kloner, 2011). Ejection fraction is one of the parameters that

allow to monitor the development of heart failure, as its reduction is associated with a poor prognosis (Cohn et al., 1993). The magnitude of remodeling correlates well with infarct size (Anversa et al., 1991). Heart failure after myocardial infarction is considered one of the main causes of cardiovascular morbidity and mortality (Cohn et al., 2000).

1.2- Myocardial stunning

Myocardial stunning can be defined as a state of transient depression of contractile function occurring after a short episode of myocardial ischemia, in spite of normal perfusion and absence of necrosis (Heyndrickx et al., 1975; Heyndrickx et al., 1978). This phenomenon can be observed both in the clinical scenario and in experimental studies. Ischemia causes cessation of cardiac contraction within few minutes after oxygen deprivation (Kloner and Jennings, 2001). The length of the time to recover ventricular function depends on the duration of the ischemic insult, the severity of ischemia and the adequacy of reperfusion (Kloner et al., 1983).

The mechanisms contributing to myocardial stunning are not completely understood, although previous studies have suggested that around 50% or 70% of cardiac dysfunction is due to ROS release during the first few minutes of reperfusion (Bolli et al., 1989). In this regard, administration of ROS scavengers before reperfusion has been shown to attenuate stunning in experimental studies (Myers et al., 1985; Przyklenk and Kloner, 1986). Other possible factors involved in the genesis of myocardial stunning include alterations in Ca^{2+} availability and in the sensitivity of the contractile apparatus to Ca^{2+} (Gao et al., 1997). Probably, both mechanisms are related because ROS release during reperfusion can damage membrane ionic channels, reducing Ca^{2+} availability, and can also alter troponin, decreasing myofilament sensitivity to Ca^{2+} (Kloner and Jennings, 2001). However, clinical trials with ROS scavengers and other drugs used to reduce myocardial stunning have not been totally conclusive, except for a decrease in reperfusion arrhythmias. Therefore, from a clinical point of view, it seems more relevant to reduce cell death than myocardial stunning (Pomblum et al., 2010).

1.3- Increased arrhythmogenesis

The mechanisms involved in the genesis of ventricular arrhythmias, which include tissue abnormal automaticity, triggered activity and re-entry, are present during

ischemia-reperfusion. Abnormal automaticity is the property of spontaneous impulse initiation, generated in myocardial fibers that are partially depolarized by pathological processes, in this case, ischemia-reperfusion. They are dependent on alterations on several ionic currents, mainly affecting phase four of transmembrane action potentials (Carmeliet, 1999). Triggered activity occurs on the top of afterdepolarizations, transmembrane voltage oscillations that interrupt the process of repolarization of a preceding impulse. Early afterdepolarizations originate during the plateau or final repolarization, while delayed afterdepolarizations originate later after the preceding action potential. When afterdepolarizations reach a threshold, a triggered activity is developed. Both types are mostly due to alterations in Ca^{2+} currents, although other mechanisms might be also involved, including catecholamine release and mechanical stretch occurring at the border zone surrounding the ischemic area (Janse and Wit, 1989). Finally, re-entrant excitation can result from circus movement or reflection, with the former being the most common mechanism. Circus movement consists on impulse propagation that traverses a re-entrant loop. The path for the circus movement usually must have adequate central and lateral boundaries, its length must exceed the wavelength determined by effective refractoriness, and there must be unidirectional block of the initiating impulse. It is usually generated in situations of heterogeneous action potential and refractory period durations, and slow conduction (Carmeliet, 1999).

Previous studies in large animal models have described an increased incidence of arrhythmias after the onset of ischemia, which mostly occur in two phases: one between 5 and 10 minutes of the beginning of ischemia (phase Ia), and the other between 15 and 30 minutes (phase Ib) (Kaplinsky et al., 1979; Euler et al., 1983; Russell et al., 1984; Cinca et al., 1997). Although these phases have been described in animal models, it is thought to be similar in humans, as most of the episodes of ventricular fibrillation (VF) and sudden death occur shortly after the onset of ischemia (de Groot and Coronel, 2004). Ischemia is associated with metabolic and ionic changes, including K^+ efflux from the cell and accumulation of metabolites at the extracellular space. This leads to cell membrane depolarization, slowing conduction and enhancing refractoriness. The heterogeneous distribution of these electrophysiological alterations is the main cause of phase Ia arrhythmias, that are maintained mainly by re-entrant mechanisms, and occur in the ischemic border zone (Janse et al., 1979; Sedlis, 1992). However, focal mechanisms may also contribute to this first phase of arrhythmias (Russell et al., 1984). During the following 15 minutes of ischemia, extracellular K^+

concentrations become more stable, but they rise again several minutes later. This phenomenon, together with cell uncoupling, Ca^{2+} overload, catecholamine secretion and adenosine triphosphate (ATP) depletion seem to be responsible for occurrence of early and delayed afterdepolarizations, which cause arrhythmias during phase Ib (Cascio et al., 1995). Finally, if the ischemic period is prolonged for more than two hours, there is an increased incidence of arrhythmias due to abnormal automaticity or triggered activity in surviving Purkinje cells (phase II) (Clements-Jewery et al., 2005).

The incidence and severity of ventricular arrhythmias during reperfusion depends on the duration of ischemia, the existence of previous ischemic episodes, heart rate, and the size of the area at risk (ischemic area), together with the presence of drugs (Coronel et al., 1992). Arrhythmias during reperfusion following short periods of ischemia (less than 10 min in pigs) are usually accelerated idioventricular rhythms and ventricular tachycardia (VT), that occur soon after reperfusion, and that may degenerate into VF. After longer periods of ischemia (20 or 30 minutes in pigs), reperfusion arrhythmias appear in two phases. The early phase starts just at the beginning of reperfusion, when Ca^{2+} overload favours the appearance of triggered activity in the reperfused zone, and that may degenerate rapidly into re-entrant VT or VF. The delayed phase of arrhythmias takes place between 2 and 7 minutes after the onset of reperfusion and they are mostly premature ventricular beats (PVBs) and VT (Figure 1), probably originated by enhanced automaticity in surviving Purkinje fibers in the area at risk (Carmeliet, 1999).

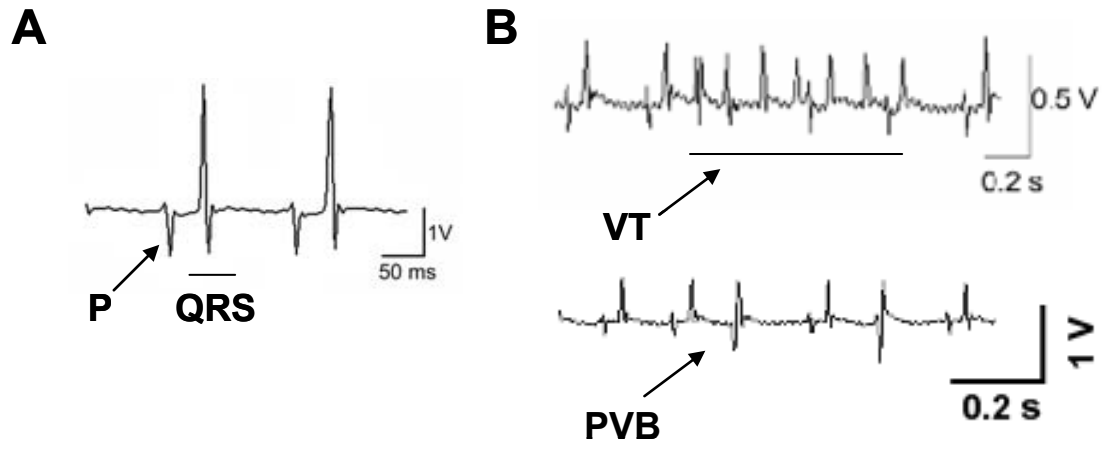


Figure 1.- Representative ECG recordings obtained in isolated mice hearts. A, ECG recording showing two cardiac cycles. Depicted are the corresponding P-wave and QRS interval. B, a short run of VT (top), and a PVB (bottom), both occurring during reperfusion. Modified from Maass et al, 2009.

2. Cellular mechanisms of myocardial ischemia-reperfusion injury

Cell death after ischemia-reperfusion occurs mainly by necrosis, although it has been proposed that it may take place by apoptosis as well (Halestrap et al., 2004; Eefting et al., 2004). It is generally accepted that sarcolemmal rupture (necrosis) is the main cause of most of cell death secondary to ischemia-reperfusion, and that it occurs during the first minutes of reperfusion (Piper et al., 1998). Previous studies have identified two major mechanisms of early cell death following ischemia-reperfusion: one of them involves hypercontracture and the consequent mechanical sarcolemmal rupture, and the other one focus on mitochondrial dysfunction. However, both mechanisms might be not completely independent, and in fact they may be interconnected.

2.1- Hypercontracture and sarcolemmal disruption

Hypercontracture develops during the first minutes of reperfusion and is manifested, in isolated cardiomyocytes, as a severe shortening caused by the strong activation of the contractile machinery. This, in cells fragilized by proteolytic activation, induces cell membrane rupture in the myocardium and can be histologically detected as areas of contraction band necrosis (Ganote, 1983; Miyazaki et al., 1987; Garcia-Dorado et al., 1992a).

2.1.1- Loss of cationic homeostasis during ischemia and reperfusion

During the first minutes of ischemia, cessation of oxygen supply to the cell causes blockade of electron transport at the inner mitochondrial membrane and a reduction in intracellular ATP levels. This, in turn, inhibits activity of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Vrbjar et al., 1993; Tran et al., 2009), and of the sarcoplasmic Na^+/K^+-ATPase, leading to an increase in cytosolic Na^+ concentration (ten Hove et al., 2007). At the same time, cessation of oxidative phosphorylation induces accumulation of end products of anaerobic metabolism, leading to an increase in both intracellular and extracellular osmolarity and to a reduction in pH (Cascio et al., 1995). Cytosol acidification triggers the activity of the Na^+/H^+ exchanger (NHE) and

Na^+/HCO_3^- co-transporter (NBC), in order to eliminate excess of H^+, inducing further Na^+ overload (Bauza et al., 1995). As a consequence of intracellular Na^+ overload, Na^+/Ca^{2+} exchanger (NCE) is activated in its reverse mode, inducing intracellular Ca^{2+} overload (Schafer et al., 2001), which leads to enzyme activation and cytoskeletal fragility (Ruiz-Meana et al., 1995). Acidosis in the ischemic period prevents, as will be discussed later, opening of mitochondrial permeability transition pore (MPTP), and together with the high Ca^{2+} concentrations, keeps GJ in a closed state. During ischemia takes place the phenomenon known as ischemic rigor contracture, denoted in isolated hearts as an increase in left ventricular end-diastolic pressure (LVEDP) due to failure of myofibril relaxation, as a consequence of ATP depletion (Allshire et al., 1987).

When coronary flow is restored, oxygen resupply activates oxidative phosphorylation, increasing ATP levels. This activates SERCA, leading to sarcoplasmic reticulum Ca^{2+} overload. However, sarcoplasmic reticulum capacity is limited and when it reaches a maximum, Ca^{2+} is released, generating a series of cytosolic Ca^{2+} oscillations (Piper et al., 1998), that together with elevated ATP levels cause hypercontracture of myofibrils (Piper et al., 2006). Simultaneously, intracellular acidosis is rapidly normalized through the activity of NHE and NBC, thus removing an inhibitor of hypercontracture, and increasing further Na^+ overload (van Borren et al., 2004). Damage of the Na^+/K^+-ATPase due to activation of enzymes like calpains (Inserte et al., 2005) impairs removal of Na^+, which leads to activation of the NCE in its reverse mode, further increasing Ca^{2+} overload, and contributing to hypercontracture (Schafer et al., 2001) (Figure 2).

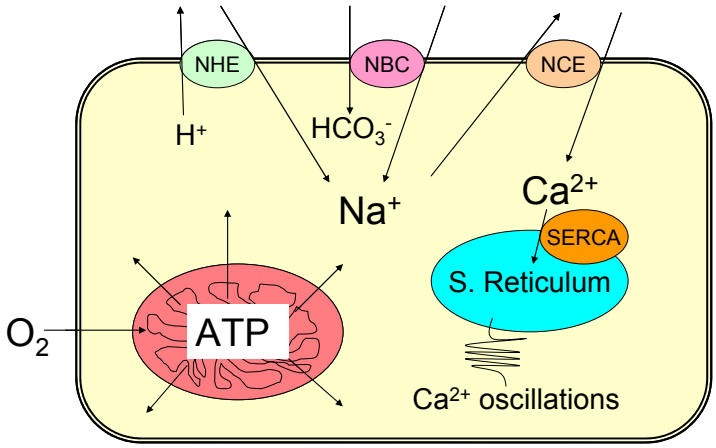


Figure 2- Scheme of major cation changes during the first minutes of reperfusion which evoke to hypercontracture and cell rupture. See text for a more detailed explanation.

2.1.2- Calpains activation

Calpains constitute a family of non-lysosomal Ca^{2+}-dependent proteases, which are activated in presence of Ca^{2+}. The first described members of this family were μ-calpain and m-calpain, expressed in all vertebrates. Later, other calpains were discovered, differing from μ and m-calpain in their absence in some vertebrates, their activity, and their Ca^{2+} dependence (Goll et al., 2003; Ravulapalli et al., 2009). Calpains are regulated by phosphorylation, phospholipid interaction or membrane translocation, protein activators, and calpastatin, their endogenous inhibitor (Goll et al., 2003). It has been reported that calpains remain inactive during ischemia, mainly due to cytosol acidification. However, conditions for their activation are fulfilled at the beginning of reperfusion, when Ca^{2+} concentrations are high (Yoshida, 2000). Activation of calpains at reperfusion, after recovery of intracellular pH, and due to Ca^{2+} overload, causes degradation of α-fodrin (a structural cytoskeleton protein) and ankyrin (an anchorage protein that links Na^+/K^+-ATPase to cytoskeleton) (Hernando et al., 2010). The later impair Na^+/K^+-ATPase function during early reperfusion, which contributes to intracellular Na^+ overload and, eventually, to hypercontracture. Moreover, degradation of cytoskeleton proteins at reperfusion onset causes sarcolemmal fragility by itself (Inserte et al., 2005).

2.1.3- Myocardial edema

After a long period of ischemia, intracellular osmolarity increases due to Na^+ overload and the accumulation of other end products of anaerobic metabolism. However, when coronary flow is restored, there is a rapid wash out of metabolites present in the interstitial space, thus generating an osmotic gradient between the intra- and extracellular compartments, and forcing water to enter into the cell to revert such osmotic gradient. Cellular water uptake causes intracellular edema and increases intracellular pressure originating mechanical stretch to the sarcolemma (Piper et al., 1998; Garcia-Dorado et al., 2012). Mechanical stretch is not enough to induce sarcolemmal rupture by itself, as demonstrated in isolated cardiomyocytes submitted to osmotic stress under normoxic conditions (Ruiz-Meana et al., 1995). However, when it occurs in combination with excessive cardiomyocyte contraction and sarcolemmal fragility, intracellular edema may contribute to sarcolemmal rupture and cell death (Garcia-Dorado et al., 1992b; Piper et al., 1998).

2.2- Mitochondrial dysfunction

Cardiomyocyte requirements of ATP are very high in order to maintain cardiac contraction. This turns mitochondria into very important organelles for myocardial pathophysiology. Thus, any possible treatment or situation in which mitochondria are affected will probably alter cardiac function (Sinatra, 2009). It has been previously mentioned the relevance of mitochondrial-dependent sudden energy recovery at the beginning of reperfusion, as one of the triggers of hypercontracture. However, mitochondria have been involved in cell death, by either apoptosis or necrosis, through other mechanisms. In apoptosis, or programmed cell death, mitochondrial membrane permeabilization allows the passage of pro-apoptotic proteins (Niizuma et al., 2010; Fulda et al., 2010). During ischemia-reperfusion, reactive oxygen species (ROS) formation and MPTP opening contribute to cell death by necrosis.

2.2.1- ROS formation

ROS are physiologically generated as a by-product of mitochondrial respiration in small amounts. Molecular oxygen is able to capture one electron generating superoxide (O_2^-). Complex I and III are considered responsible for much of superoxide formation. Superoxide is dangerous because it can inactivate proteins with iron-sulphur centers, such as proteins involved in mitochondrial metabolism. To avoid damage of proteins, organisms have several mechanisms of detoxification, as superoxide dismutase enzymes, which generate hydrogen peroxide (H_2O_2) from superoxide. However, H_2O_2 can interact with molecules with Fe^{2+} generating hydroxyl radicals (OH^-), which are very harmful because they can damage proteins, DNA and lipids (Raha and Robinson, 2000). In contrast, small concentrations of H_2O_2 seem to be beneficial, acting as an important second messenger and activating transcription factors (Giorgio et al., 2007).

Metabolic changes occurring during the ischemic period, such as electron transport chain uncoupling and cytochrome oxidase inactivation, together with the rapid influx of oxygen to the mitochondria at reperfusion onset, generate an abrupt increase in ROS formation during this phase (Chen et al., 2008). The increase in ROS formation has multiple effects, as oxidation of lipids, thus increasing membrane fragility, proteins, causing impairment of normal contraction and metabolism, and DNA, inducing apoptosis. Moreover, ROS damage mitochondrial respiratory chain, which also feeds back its own production (Becker, 2004). It has been also described that ROS induce

MPTP opening (Di Lisa and Bernardi, 1998) and generate an inflammatory response mediated by neutrophils and cytokines, which, in turn, may also induce further ROS formation (Nian et al., 2004; Vinten-Johansen, 2004).

2.2.2- MPTP opening

MPTP is a multiprotein megachannel connecting the mitochondrial matrix with the cytosol, which allows the passage of molecules with molecular weight lower than 1.5 KDa. The molecular structure of this pore is not known. Previous studies agreed about the involvement in this complex of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and cyclophilin-D (CyP-D) (Crompton, 1999). Later, studies performed in knock out mice disagreed with this structural role for VDAC and ANT, although a regulator effect of ANT has been described (Halestrap, 2009). MPTP opens in situations of mitochondrial Ca^{2+} overload, especially when it is accompanied by oxidative stress, ATP depletion and mitochondrial depolarization. Opening MPTP induces mitochondrial edema and depolarization, oxidative phosphorylation uncoupling and the release of pro-apoptotic agents. Cytosol and mitochondrial matrix acidification avoid MPTP opening, therefore keeping it closed during ischemia, while changes at the beginning of reperfusion, mainly pH recovery and Ca^{2+} overload, induce MPTP opening (Halestrap et al., 2004).

The role played by MPTP opening in cell death during reperfusion has been analyzed using cyclosporine-A (CsA) and sanglifehrin A, two drugs inhibiting MPTP opening through its binding to CyP-D. The administration of both drugs resulted in a reduced infarct size in isolated rat hearts (Di Lisa et al., 2001; Hausenloy et al., 2003; Argaud et al., 2005). The role of MPTP opening in reperfusion injury was later confirmed in CyP-D deficient mice, which were protected against ischemia-reperfusion injury (Baines et al., 2005).

There is some discussion about which mechanism is more important for reperfusion injury: hypercontracture or MPTP opening. In fact, both mechanisms of cell death might be interconnected. On the one hand, MPTP opening may be secondary to the sarcoplasmic-dependent Ca^{2+} oscillations evoked by reperfusion (Piper et al., 2006). But, on the other hand, MPTP opening may induce hypercontracture of isolated, Ca^{2+}-overloaded, cardiomyocytes (Ruiz-Meana et al., 2007). Duration of ischemia seems to be crucial in the way by which the cell dyes, as hearts from CyP-D deficient mice were

protected from reperfusion injury only after long periods of ischemia but not after shorter ischemias (Ruiz-Meana et al., 2011).

3. Endogenous mechanisms of myocardial protection

Despite the serious consequences of myocardial ischemia-reperfusion injury, or perhaps, thanks to them, multicellular organisms have evolved several mechanisms of endogenous protection, including local and remote ischemic pre- and postconditioning. A large number of stimuli able to trigger these states of increased resistance to ischemia-reperfusion have been identified, and the multiple and interrelated signal transduction pathways downstream of pre- and postconditioning triggers have been elucidated to a large extent (Garcia-Dorado, 2004; Cohen and Downey, 2011). However, the end-effectors of protection, linking the long chain of events that starts with triggers, continues with mediators, and ends with protection, remain largely unknown.

3.1- Ischemic preconditioning

IPC, first described by Murry et al. in 1986, consists of brief, sublethal, episodes of ischemia-reperfusion that protect the myocardium against necrosis induced by a subsequent ischemia-reperfusion (Murry et al., 1986). The anti-infarct effect of IPC has been reproduced in all animal models tested and even in man. More recent studies have demonstrated the existence of two different phases of IPC (Schulz et al., 2001). While the first window or early phase of protection is short-lived, a second window or late phase of protection appears about 24 h after the preconditioning stimulus. This second window of protection is believed to last for up to 2-3 days. Although the signal transduction pathway leading to protection shares many steps in both phases, there are certain differences between them. Nitric oxide (NO) seems to be a key trigger and mediator in the late phase of IPC, which involves altered gene expression (Yellon and Downey, 2003).

For the occurrence of the classical or early phase of protection, a critical threshold needs to be exceeded. A preconditioning stimulus of more than 2 min of ischemia with subsequent reperfusion prior to prolonged ischemia is required to obtain the protective effect in several species (Schulz et al., 2001). Once this requirement is achieved, protection may last for a period of about 2-3 h. Beyond this period, protection is lost.

A large number of studies have identified several stimuli able to trigger IPC protection against ischemia-reperfusion injury, and signal transduction pathways involved in this protection have been, in part, elucidated. However, the end-effectors involved in IPC are not completely known (Garcia-Dorado et al., 2006). Several receptor-dependent and receptor-independent endogenous triggers have been suggested to play a role in initiating the protective response. Among receptor-dependent triggers, adenosine, bradykinin and opioids, depending on animal species, seem to play a preponderate role, but also prostaglandins, norepinephrine, angiontensin or endothelin may be important in some situations (Schulz et al., 2001; Yellon and Downey, 2003). ROS and NO might be involved in receptor-independent triggering (Schulz et al., 2001; Yellon and Downey, 2003). Activation of G protein-coupled receptors (GPCR) by its endogenous ligands causes a conformational change in the receptor, promoting coupling and activation of heterotrimeric $G_{\alpha\beta\gamma}$ proteins. In the case of receptor-dependent triggers, activation of $G_{\alpha q/11}$ by most GPCRs results in the hydrolysis of membrane bound phosphatidylinositol 4,5-biphosphate by phospholipase Cβ, releasing inositol 1,2,5-triphosphate (IP3) and diacylglycerol. The last one functions as a protein kinase C (PKC) activator. On the other hand, binding of ligands to its GPCR also leads, probably through the βγ subunits of the G proteins, to serial activation of the reperfusion injury salvage kinases (RISK) pathway (Schulz et al., 2001; Yellon and Downey, 2003). It includes phosphatidylinositol 3-kinase (PI3K), Akt, extracellular signal-regulated protein-1/2 (ERK-1/2), NO synthase (NOS), and guanylyl cyclase (GC), which ends-up increasing intracellular cyclic guanosine monophosphate (cGMP) levels. This, in turn, activates protein kinase G (PKG) during the trigger phase (Cohen and Downey, 2011). Receptor-independent triggers interact with several steps of this cascade of events. Finally, all these intracellular signaling pathways converge at the mitochondrial level, probably through actions on the mitochondrial ATP sensitive potassium channels ($mitoK_{ATP}$) and/or MPTP (Miura et al., 2010b), which lead to a small burst of ROS and activation of PKC (Downey et al., 2007). Importantly, IPC can be mimicked pharmacologically by exogenous application of the above mentioned triggers (Peart et al., 2003), and can be also induced by short episodes of ischemia-reperfusion distant to the target organ (Hausenloy and Yellon, 2008).

An important feature of IPC protection is that it is independent of slowed energy depletion during ischemia. In fact, it is manifested during subsequent reperfusion.

During this phase, several mediators have been described to play a role in protection, with a possible involvement, again, of the RISK pathway (Hausenloy et al., 2005) (Figure 3). In contrast, the final effectors of protection have not been fully elucidated, although several candidates have been proposed, including attenuated Ca^{2+}-dependent hypercontracture, reduced calpain activity, attenuated cell-to-cell propagation of necrosis and reduced MPTP opening (Garcia-Dorado et al., 2006).

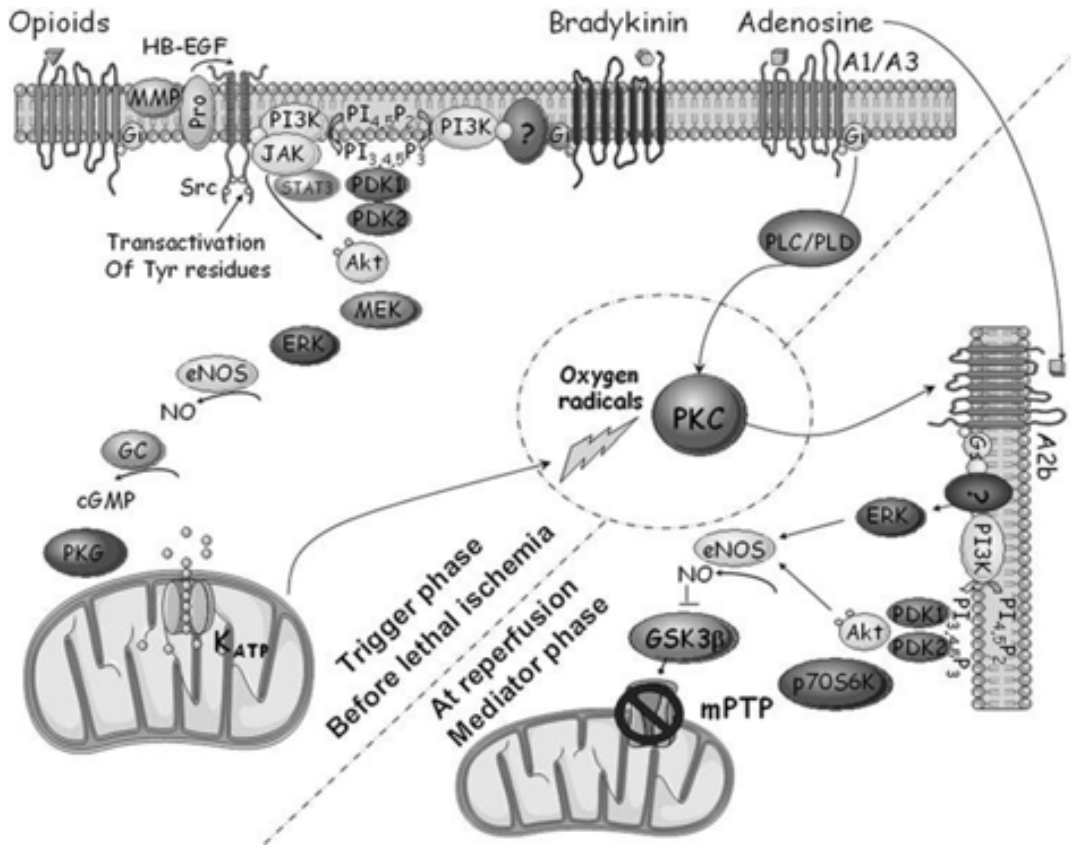


Figure 3.- Scheme proposed for IPC signaling pathway (RISK pathway). See text for details (Cohen and Downey, 2011).

Some authors have postulated the involvement in IPC protection, at least under some conditions, of a different signaling pathway, the survivor activating factor enhancement (SAFE) pathway, which includes activation of the Janus kinase (JAK), and the signal transducer and activator of transcription-3 (STAT3). It has been reported that STAT3 protects from ischemia-reperfusion modifying the expression of pro-apoptotic genes and phosphorylating glycogen synthase kinase 3β (GSK3β) (Lecour, 2009). A cross-talk between RISK and SAFE pathways cannot be excluded, as inhibition of one of those signaling cascades affects the other one (Suleman et al.,

2008). In this way, NO is involved in both signaling cascades, as a mediator in the RISK pathway, and as a trigger in the SAFE one, respectively (Heusch et al., 2008).

Clinical application of IPC, or pharmacological preconditioning by exogenous triggers, is hampered by the fact that it should be applied before ischemia. This practically restricts its potential application to cardiac surgery (Yellon and Downey, 2003). In this sense, IPC has been shown to reduce troponin-T release in patients undergoing elective coronary artery bypass graft, a finding suggestive of reduced infarctions (Jenkins et al., 1997). Furthermore, remote IPC by short episodes of upper or lower limb ischemia-reperfusion has been shown also to reduce release of troponin-T in adult or pediatric patients receiving cardiac surgery (Cheung et al., 2006; Hausenloy et al., 2007).

3.2- Ischemic postconditioning

Ischemic postconditioning was first described by Zhao et al. in 2003, and consists in the application of very short episodes of ischemia-reperfusion just at the beginning of reperfusion, which confers, depending on the conditions, a similar protection to IPC (Zhao et al., 2003). Ischemic postconditioning opens a new window for reducing infarct size in patients with acute myocardial ischemia, as it can be easily applied during percutaneous coronary interventions. In fact, preliminary studies in patients with acute myocardial infarction submitted to percutaneous revascularization have shown promising results (Staat et al., 2005; Thibault et al., 2008), that await confirmation in larger clinical trials.

Initial lines of evidence suggested that the RISK pathway was the signaling cascade involved in protection by ischemic postconditioning (Tsang et al., 2004). In fact, it has been suggested that protection by ischemic postconditioning shares some of the intracellular signaling pathways of IPC. However, recent studies have demonstrated in *in situ* pig hearts that protection by ischemic postconditioning can be achieved without activation of the RISK pathway (Skyschally et al., 2009; Heusch et al., 2011). Moreover, the demonstration that the SAFE pathway was also activated by ischemic postconditioning confirmed the existence of other possible mechanisms (Lacerda et al., 2009). One of such additional mechanisms can be a delay in intracellular pH recovery during reperfusion. Postconditioning has been demonstrated to prolong acidosis during reperfusion (Inserte et al., 2009), which has inhibitory effects on hypercontracture,

enzyme activation and MPTP opening. Nuclear magnetic resonance (NMR) spectroscopy studies in isolated rat hearts suggest that most, if not all, the protective effect of postconditioning is explained by this effect (Inserte et al., 2009). Further studies are needed to completely elucidate the mechanisms of protection by ischemic postconditioning (Ovize et al., 2010). Interestingly, postconditioning can be also mimicked pharmacologically (Jin et al., 2007), and can be applied in remote areas (Li et al., 2006). Development of pharmacological tools mimicking postconditioning protection is of great importance as they could be used in patients reperfused by thrombolytic treatment, which otherwise would be not amenable to mechanical postconditioning.

3.3- Remote ischemic conditioning

Application of brief episodes of ischemia-reperfusion not only confers protection against reperfusion injury in the same tissue where they are applied, but they can also protect distant tissues (Przyklenk et al., 1993), in a process known as remote ischemic conditioning. Previous studies have suggested that humoral factors may mediate this benefit, as perfusion of control isolated hearts with the coronary effluent collected from donor-hearts submitted to IPC cycles, is able to induce protection against ischemia-reperfusion injury in the transfused hearts (Dickson et al., 1999). The cardioprotective factors released in the coronary effluent have been found to activate opioids receptor and PKC, thus indicating that remote ischemic conditioning probably shares some molecular mechanisms with IPC (Serejo et al., 2007; Shimizu et al., 2009). Interestingly, although it was first described as a protective strategy performed before ischemia, a reduction in infarct size has also been achieved in rats, when remote ischemic conditioning was performed during ischemia, just before reperfusion (Kerendi et al., 2005). Furthermore, applicability of this manoeuvre to humans was demonstrated after application of transient limb ischemia which induced protection against endothelial dysfunction in the contralateral arm or leg caused by a more prolonged ischemic insult (Loukogeorgakis et al., 2007). The clinical applicability of ischemic conditioning to patients undergoing myocardial infarction has been recently shown in a clinical study in which four cycles of five minutes of cuff inflation and deflation to the arm, performed during ambulance transportation, attenuates reperfusion injury (Botker et al., 2010). However, larger clinical trials are needed to confirm these results. The importance of

ischemic conditioning is based on the fact that it is a non-invasive and traumatic technique which could be easily performed as a routine to ameliorate myocardial injury.

To summarize, the molecular mechanisms involved in myocardial ischemia-reperfusion injury and in endogenous cardioprotection have been widely studied during the last years, but are not completely understood. This thesis tries to clarify the role played by Cx43 in both situations.

4. Connexins and gap junctions

4.1- Structure and location of connexins

Connexins constitute a large family of sarcolemmal proteins sharing a common structure, consisting in four transmembrane α-helices, two extracellular and one intracellular loop, and amino and carboxy-terminal domains located at the cytoplasmic side of the membrane (Harris, 2001). At least 21 members of this family, which are distinguished by their theoretical molecular weight (in KDa), have been described in humans (Sohl and Willecke, 2003). Differences between these connexin isoforms are mostly due to variations in amino acid sequence at the carboxy-terminal domain, although differences in the cytoplasmic loop have been also described (van Veen et al., 2001). The carboxy-terminal domain has multiple serine, threonine and tyrosine residues susceptible of phosphorylation, which are important for connexin regulation (Kemp and Pearson, 1990; Bevans and Harris, 1999). Actually, 19 phosphorylation sites have been described for Cx43. Among them, serine 368 is the most studied, but serines 296, 297, 306, 325, 328 and 330 are also of importance in trafficking, assembly or regulation (Procida et al., 2009). The phosphorylation state of these residues is responsible for the appearance of multiple bands in western blots.

Six molecules of connexin leaving a central pore form a hemichannel or connexon, which can be made up by the same connexin isoform (homomeric connexons) or by different isoforms (heteromeric connexons). Interaction between the extracellular domains of two hemichannels from adjacent cells originates an intercellular channel, which tend to aggregate in plaques, known as GJ (van Veen et al., 2001) (Figure 4). In the process of docking of two hemichannels, are important three conserved cysteines residues located in each extracellular loop of each connexin molecule. Disulfide bonds between these cysteines are needed to create the β-sheet conformation required for the interaction between the two opposing hemichannels. Gap junctional plaques are formed in areas in which membranes of neighbouring cells are in close contact, known as intercalated disks, and that in cardiomyocytes are located at the cell poles, perpendicular to the longitudinal axis of the cells (Shaw et al., 2007).

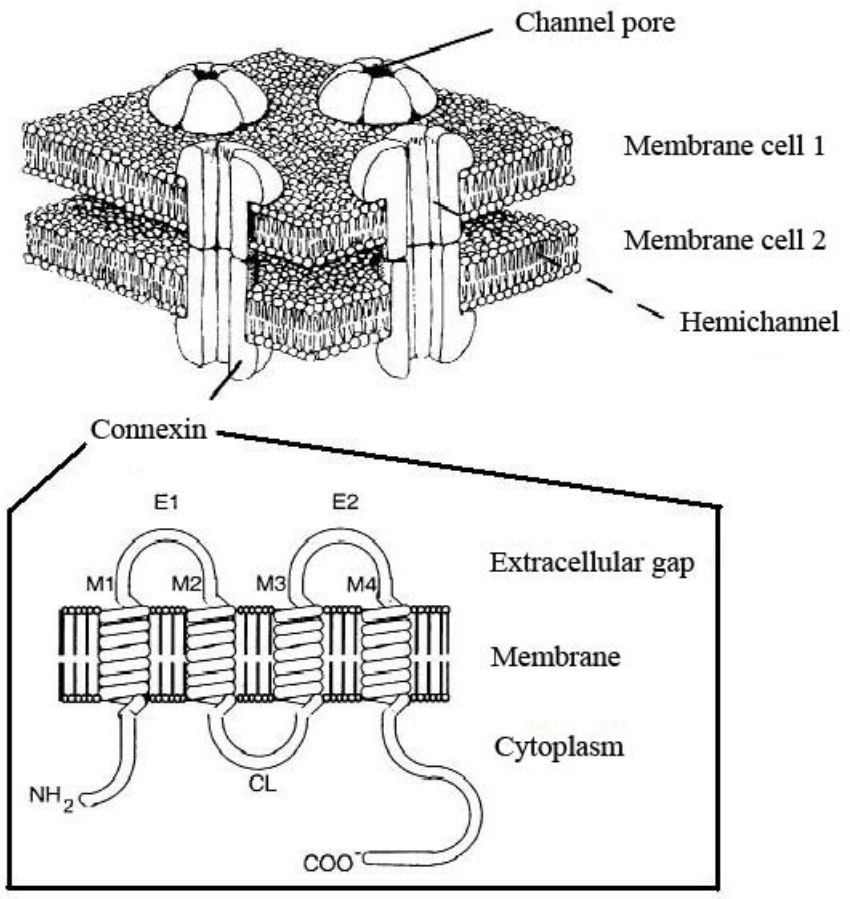


Figure 4.- GJ structure. The upper image shows a gap junctional plaque with their intercellular channels connecting two adjacent cells. The lower image shows the structure of a connexin molecule (Modified from van Veen et al., 2001).

GJ intercellular channels put into contact the cytoplasm of two neighbouring cells, thus mediating both electrical and metabolic coupling. They allow the passage of molecules with molecular mass up to 1.2 KDa, such as sugars, signaling mediators and ions (van Veen et al., 2001). Both electrical and metabolic coupling are essential for many tissues. In the heart, GJ mediate synchronized cardiac contraction (Jalife et al., 1999), whereas in other tissues they are important for cell growth and differentiation, embryonic development and tissue homeostasis (Kjenseth et al., 2010).

Connexins are synthesized in the endoplasmic reticulum, but they oligomerize into hemichannels in the Golgi complex. Connexons are then transported into vesicles to the cell membrane, in a process in which microtubules and actin filaments are essential (Kjenseth et al., 2010). Although this process is not completely understood, recent evidences indicate that hemichannels might be directly delivered to the intercalated disks (Shaw et al., 2007).

Intercalated disks can be seen, in fact, as an "organelle" involved in maintaining synchrony within cell populations, in which desmosomes and adherens junctions give mechanical continuity, whereas GJ provide a pathway for transfer of ions and small molecules between cells. In this way, connexins located at this "organelle" interact with a number of other proteins, either junctionals or non-junctionals, including sodium channels, microtubules, and proteins from mechanical junctions, as cadherins, plakophilin or plakoglobin. Some of these proteins might be important for GJ function. In fact, formation of functional GJ requires pre-formation of mechanical junctions (Saffitz, 2006; Rohr, 2007). In this sense, inhibition of cadherin expression has been described to disrupt GJ assembly (Wei et al., 2004). Moreover, Zonula Occludens -1 (ZO-1), which links cadherin to the actin cytoskeleton, may mediate localization of both cadherins and GJ at the intercalated disks (Palatinus et al., 2011), and a direct or indirect cross-talk between the carboxy-terminal domain of connexins and ZO-1 has been suggested to regulate GJ assembly (Chakraborty et al., 2010).

Turnover of connexins is rapid, with some connexin isoforms having very short half lifes (1.5-5 hours). Ubiquitination is an important regulatory mechanism of connexins degradation, which can rapidly modify the number of GJ present at the cell membrane. After ubiquitination, connexins are removed from GJ by endocytosis in one of the cells, forming a double-membrane vacuole called annular GJ, which is then transformed into an endosome. In this state, connexins might be recycled and transported again to the plasma membrane forming hemichannels, or may be degraded in lysosomes (Kjenseth et al., 2010) (Figure 5). The knowledge of the molecular mechanisms involved in connexins biosynthesis, GJ assembly and their degradation is important to find indirect methods able to modify their abundance.

In addition to their presence at intercalated disks, connexins are also located in other regions of the plasma membrane as single hemichannels. In this way, they may allow the exchange of molecules and ions between the extracellular space and the cytoplasm (Rodriguez-Sinovas et al., 2012). First, it was thought that these hemichannels should be kept closed to maintain cellular homeostasis, and that they opened only under pathological conditions. However, it is now well known that they are strictly regulated, and that their opening controls important functions (Saez et al., 2010). Extracellular cation concentrations, intracellular pH, transmembrane potential and mechanical stimulation, among others, have been found to modulate hemichannel gating. As most of these factors are altered during ischemia, hemichannel opening has

been proposed to play a role in ischemia-reperfusion injury by increasing intracellular Ca^{2+} overload and edema (Shintani-Ishida et al., 2007).

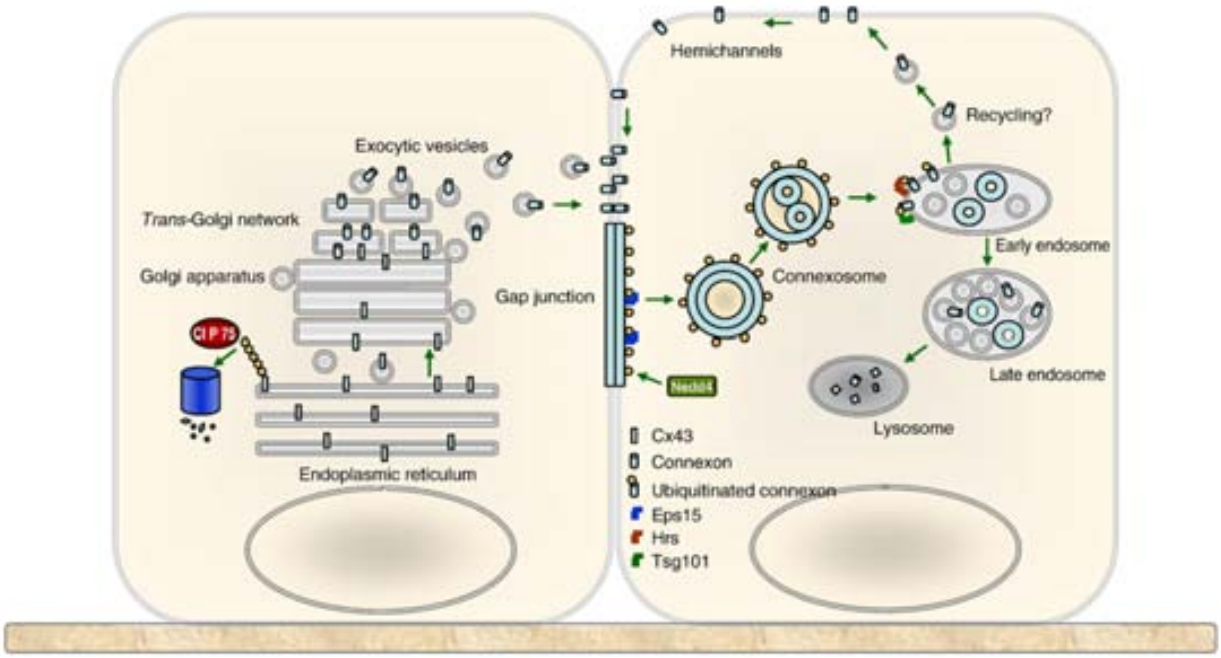


Figure 5.- Intracellular trafficking of connexins. On the left cell, GJ synthesis and transport from endoplasmic reticulum to the intercalated disk is shown, and, on the right one, ubiquitination-mediated connexin degradation (specifically Cx43) is depicted (Kjenseth et al., 2010).

Although plasma membrane is the main final destination for most of connexin molecules, localizations other than cell membrane have been also reported for some isoforms. In fact, the entire Cx43 protein (Huang et al., 1998) or its carboxy-terminal domain (Dang et al., 2003) have been found in the nucleus of human brain glioblastoma tumor cells, and in cardiomyocytes and HeLa cells, respectively. There, connexins may directly affect gene transcription. Thus, studies in Cx43-defficient cells have demonstrated altered expression of several apoptotic genes, as those coding for some caspases (Vinken et al., 2012). Furthermore, Cx43 overexpression in neonatal rat cardiomyocytes has been shown to decrease DNA synthesis, by mechanisms independent of GJ intercellular communication (Doble et al., 2004). Moreover, several lines of evidence have supported that only the carboxy-terminal domain of some connexin isoforms is responsible for their regulatory effect on cell growth, as this effect is lost after truncation of the carboxy-terminal tail (Omori and Yamasaki, 1999). This finding is also supported by the fact that mutations in the extracellular loop of Cx43

abolished GJ formation, but did not modify the inhibitory effect on cell proliferation (Olbina and Eckhart, 2003). As occurred with Cx43, other connexin isoforms, as connexin 45.6, have been described to promote lens cell differentiation, through a mechanism independent of GJ communication (Gu et al., 2003).

Interestingly, the presence of Cx43 in mitochondria from endothelial cells (Li et al., 2002b) and cardiomyocytes (Boengler et al., 2005; Rodriguez-Sinovas et al., 2006a) has been recently described. The mitochondrial location of Cx43 has been later confirmed in brain (Azarashvili et al., 2011), astrocytes (Kozoriz et al., 2010) and bone marrow stem cells (Lu et al., 2010). Translocation of Cx43 to the inner membrane of cardiomyocyte mitochondria has been described to occur through the translocase of the outer mitochondrial membrane (TOM) and translocase of the inner mitochondrial membrane (TIM) system, in a heat shock protein-90 (Hsp-90)-dependent manner (Rodriguez-Sinovas et al., 2006a). Although functions of mitochondrial Cx43 remain uncertain, it has been recently suggested that it may play a role in controlling mitochondrial potassium uptake by forming hemichannels at the inner mitochondrial membrane of cardiomyocytes (Miro-Casas et al., 2009) and astrocytes (Kozoriz et al., 2010).

4.2- Connexin isoforms. Expression patterns

Connexins, as a large family of transmembrane proteins involved in essential cell functions, are expressed in almost all tissues, except for differentiated skeletal muscle, erythrocytes and mature sperm cells (Rackauskas et al., 2010). As mentioned before, 21 different connexin isoforms have been described in humans, although not all of them are expressed in all tissues.

Some cell types, as keratinocytes, express a wide variety of connexin isoforms, including Cx43, connexin 26, 30, 31, 40 and 45, whereas others, as hepatocytes, express a limited diversity of connexin isoforms, including only connexin 32 (Cx32) and 26 (Rackauskas et al., 2010). In the other extreme, connexin 57 is the only isoform that has been described in horizontal retinal cells, although other unknown connexin might be also expressed there, as connexin 57-null mice do not have apparent sight or behaviour defects (Hombach et al., 2004). However, Cx43 is by far the most widely expressed connexin isoform, present at least in 34 tissues and 46 different cell types, including cardiomyocytes and astrocytes (Laird, 2006). Although four connexin isoforms (Cx43,

connexin 40, 45 and 30.2 in mice, and 31.9 in humans) have been described in the heart, only Cx43 and connexin 40 are mostly expressed in the working myocardium, with Cx43 being by far the most abundant. In fact, GJ formed by both isoforms are present in atrial cardiomyocytes whereas only Cx43 forms GJ in ventricular myocardium (Lin et al., 2010).

4.3- Permeability and conductance of connexin channels

GJ-mediated intercellular coupling is determined by several factors, including the number of available channels, single channel conductance or permeability, and the open probability of a single channel (van Veen et al., 2001). GJ are permeable to relatively large molecules (Harris, 2001). Depending on the connexin isoform, pore diameter ranges between approximately 6.5 and 15 Å (Veenstra et al., 1994; Beblo and Veenstra, 1997; Harris, 2001), which is wide enough to allow the passage of water, all relevant cations and anions, including Cl^-, Na^+, K^+, and Ca^{2+}, and most second messengers, as IP3, adenosine, adenosine diphosphate (ADP), ATP, glutamate, cyclic adenosine monophosphate (cAMP), cGMP, polypeptides and small interfering RNAs (Bevans et al., 1998; Harris, 2001). For small molecules, junctional channels behave as rather unselective pores. However, as the size of the permeant increase, selectivity becomes more apparent, thus causing that different connexin isoforms differ in their permeabilities to distinct cytoplasmic molecules (Harris, 2001). Importantly, GJ permeability depends not only on the molecular size, but also on the net charge, as different fluorescent probes with similar size, but opposite charge, have been shown to differ in their permeability through GJ formed by several connexin isoforms (Kanaporis et al., 2011). Differences in selectivity are clearly illustrated in the case of GJ formed by Cx43 and Cx32. Thus, although Cx43-formed GJ channels show a maximal unitary conductance about two fold higher than those formed by Cx32, Cx43 is more cation-selective than Cx32 (Harris, 2002), with differences in permeability to adenosine, ATP, ADP and adenosine monophosphate (AMP) (Goldberg et al., 2002).

As occurred with chemical permeability, maximal unitary conductance depends on the connexin isoform. Considering those normally expressed in the heart, Cx43 has a maximal unitary conductance of about 100 pS, with connexin 45 having lower values (30 pS), and connexin 40 higher (175 pS) (Garcia-Dorado et al., 2004). Other isoforms also differ in their maximal unitary conductance, as are the case of connexin 30 (160

pS), connexin 36 (15 pS) and Cx32 (60 pS) (Harris, 2001; Rackauskas et al., 2010). Regarding GJ gating, available evidence suggest that it can take place in at least two independent ways (Bukauskas and Peracchia, 1997; Bukauskas and Peracchia, 2000; Skerrett et al., 2000; Harris, 2001; Moreno et al., 2002): a rapid, voltage driven, mechanism that can change the channel conformation between the fully open state and a nearly completely closed state within few ms (voltage gating), and a slower mechanism (up to 30 ms) that brings the channel to complete closure in response to chemical interactions (chemical gating), or to changes in voltage (loop gating). The existence of the first gating mechanism, in which GJ conductance reaches about 5 to 30% of its maximal (depending on the connexin isoform), but not zero conductance, creates what is known as residual state or substate (Bukauskas and Peracchia, 1997; Harris, 2001; Bukauskas et al., 2002).

Macroscopic GJ conductivity depends both on the unitary conductivity of open junctional channels and on the number of open channels (what is known as open probability). It is important to notice that regulatory mechanisms may have opposed effects on the average conductivity of open channels (size of the opening) and on the probability of the open state. According to this, a given stimulus may reduce the permeability to large molecules, while increasing the macroscopic conductance to small ions and enhancing electrical coupling (Kwak et al., 1995; Dhein, 1998). This would be the case that has been described for Cx43 in several cell lines, for which the residual state is more anion-selective, and allow persistence of electrical cell to cell communication, but not of metabolic coupling (Bukauskas et al., 2002). The effects of different conditions on GJ cannot be safely summarized as “increase” or “decrease” in GJ-mediated intercellular coupling, but on its consequences on permeability, selectivity and electrical coupling.

4.4- Connexin regulation

As seen in the last point, GJ-mediated intercellular coupling depends on the connexin isoform, the number of available channels, and their open probability. Besides regulation of connexin synthesis, GJ assembly and degradation, permeability and conductance of junctional channels is strictly regulated by several factors, as transmembrane potential and transjunctional voltage (Vm and Vj, respectively), and by

chemical factors, including pH, intracellular Ca^{2+} and calmodulin, phosphorylation and nucleotides.

4.4.1- Transmembrane potential and transjuctional voltage

GJ-channels are differentially regulated by voltage changes, depending on the connexin isoforms that are expressed. Some of them are influenced by Vm and Vj, whereas others only by Vj. The exact mechanisms of voltage gating are not well known, but it has been suggested that each connexin hemichannel may have at least two different gates: whereas the fast gate depends only on Vj, the slow mechanism may depend both on Vj and Vm. These two gates should be located at different structural areas of connexins, as they can be modified by mutations in different regions of the protein (Gonzalez et al., 2007). As mentioned before, the fast Vj-gating closes the channel from the fully-open state to a residual state at either voltage polarity. Depending on the connexin isoform, some hemichannels will close under negative or positive potentials on their cytoplasmic face. For instance, connexin 26, 30 and 50 close at positive voltages, but connexin 31, 32, 37, 40, 43, 45 and 57, at negatives. Interestingly, connexin 46 is able to close on both, negative and positive potentials, with different gating mechanisms (Rackauskas et al., 2010). First evidences supported that voltage polarity of the fast Vj-gating mechanism was regulated by charged residues located at the amino-terminal domain, acting as voltage sensors (Bukauskas et al., 2002). However, more recent studies have reported abolition of the fast Vj-gating after truncation of the carboxy-terminal domain of different connexin isoforms (Gonzalez et al., 2007). Thus, although it cannot be excluded that the amino-terminal domain plays a role, the most accepted mechanism for fast Vj-gating is that the flexible carboxy-terminal domain binds to the cytoplasmic loop, partially closing the pore ("chain and ball" model) (Gonzalez et al., 2007).

The slow Vj-gating mechanism, that mediates transitions from/to the residual state to/from the fully closed channel, always closes under relative negative potentials into the cell. Moreover, this mechanism has been also associated with chemical gating, as Ca^{2+}, lipophiles and lowered pH have been described to fully close slow Vj-sensitive gates from the residual substate (Bukauskas and Peracchia, 1997; Peracchia, 2004). On the other hand, depolarization of Vm increases the incidence of slow transitions that lead to a fully closed channel state (Bukauskas and Weingart, 1994). Mutational studies of Cx43 have suggested that the Vm sensor is located in a different region of the

carboxy-terminal domain than that involved in fast Vj-gating. However, interactions between both gating sensors cannot be discarded (Revilla et al., 2000).

4.4.2- Intracellular pH

Cytoplasm acidification has been shown to reduce GJ intercellular communication. However, pH sensitivity is different depending on the connexin isoform that is expressed. Therefore, an slight reduction in intracellular pH is able to partially close channels formed by Cx43, but not by Cx32, which need higher intracellular acidification levels to achieve the same degree of GJ closure (Morley et al., 1996). In fact, pH sensitivity has been analyzed for a wide variety of connexin isoforms expressed in oocyte pairs showing the following decreasing order: connexin 50>46>45>26>37>43>40>32 (Stergiopoulos et al., 1999). Sensitivity to intracellular pH seems to depend on the carboxy-terminal region of the protein, as truncation of that region abolishes pH regulation of Cx43 (Liu et al., 1993). This was later confirmed for other connexin isoforms, as connexin 37, 40 and 50, but not for connexin 45 (Stergiopoulos et al., 1999). Interestingly, these studies also observed that the carboxy-terminal domain of connexin 40 is able to regulate channels formed by truncated-Cx43 and vice versa (Stergiopoulos et al., 1999). Furthermore, ablation of the carboxy-terminal domain has been observed to eliminate the residual state of GJ channels, whereas it can be restored after co-expression of the carboxy-terminal region as a separate protein (Anumonwo et al., 2001; Moreno et al., 2002). These findings made some authors to suggest that pH/chemical gating can be dependent on a "ball and chain" mechanism, similar to that previously described for fast Vj-gating (Delmar et al., 2004). However, there is still some controversy whether pH gating can be mediated by direct protonation of some histidine residues in the carboxy-terminal domain (Trexler et al., 1999), or by indirect mechanisms through protonation of endogenous aminosulfonates, such as taurine in intact cells (Harris, 2001).

4.4.3- Intracellular calcium concentrations and calmodulin

Regulation of GJ channels by Ca^{2+} has been widely analyzed (Peracchia, 2004; Lurtz and Louis, 2007). Early studies demonstrated that intracellular injections of Ca^{2+} induced electrical uncoupling (Rose and Loewenstein, 1975; De Mello, 1975). Sensitivity of GJ channels to intracellular Ca^{2+} concentrations depends not only on the connexin isoform, but also on the cell type. However, it is still unclear whether the

effects of Ca^{2+} are direct or mediated through intracellular messengers. In this sense, several evidence indicates that Ca^{2+} probably induces GJ closure by activation of calmodulin, which may act directly as a gating particle (Peracchia et al., 2000; Peracchia, 2004). Calmodulin, a soluble acidic protein, forms complexes with Ca^{2+}, which then bind to basic amphiphilic α-helix domains. Interestingly, calmodulin has been shown to colocalize with connexins (Sotkis et al., 2001), and the interaction between several connexin isoforms and calmodulin has been previously demonstrated (Rackauskas et al., 2010), including connexin 38, 32, 37, 43, 44 and 50. Closure of GJ channels by intracellular Ca^{2+} during ischemia may play an essential role in protecting intact cells from membrane depolarization, and leakage of metabolites through GJ, by disconnecting them from damaged cells in the so-called "healing-over" process (Rackauskas et al., 2010). However, the counterpart would be an increased incidence of ventricular arrhythmias (Jalife et al., 1999).

4.4.4- Phosphorylation

Although it is complex and incompletely understood, phosphorylation is an important regulatory mechanism of connexins, that not only modifies channel molecular structure, open probability and mean open-time, but also alters connexin intracellular trafficking and assembly into gap junctional plaques. Depending on the connexin isoform, the phosphorylation site and the biochemical environment, the effect of phosphorylation can be different (van Veen et al., 2001). The carboxy-terminal domain is full of serine, threonine and tyrosine residues that can be phosphorylated by multiple protein kinases. In the case of Cx43, some of these serine residues able to be phosphorylated are: 364, phosphorylated by protein kinase A (PKA), 368 by PKC, and 255, 279 and 282 by mitogen activated protein kinases (MAPK) (Lampe and Lau, 2000). In addition, connexin 36 and 56 can be phosphorylated on the cytoplasmic loop (Rackauskas et al., 2010).

Phosphorylation may modify the net charge of the protein, which, in turn, may also alter voltage or pH sensitivity. Cx43 regulation by phosphorylation has been widely analyzed. It has been described that phosphorylation of Cx43 by PKA increases cell to cell communication and channel permeability, whereas phosphorylation by PKG and MAPK reduces GJ intercellular communication in cardiomyocytes. More controversial is the effect of phosphorylation mediated by PKC isoforms α and ε on Cx43. On one hand, it reduces single channel conductance and cell-to-cell permeability, but on the

other hand, it enhances electrical conductance between paired cardiomyocytes (Kwak and Jongsma, 1996; Schulz and Heusch, 2004). In addition, PKCε activation may induce Cx43 hyperphosphorylation, which has been shown to accelerate Cx43 proteolytic degradation, resulting in decreased number of GJ channels and conduction impairment (Lin et al., 2006). Reduced GJ intercellular communication has been also observed after protein tyrosine kinase phosphorylation of Cx43 at residue 265, that avoids binding of the protein to ZO-1, and therefore, decreases Cx43 location at the intercalated disks. Moreover, Cx43 phosphorylation of serine residues 325 and 330 by casein kinase in rat kidney cells has been shown to reduce membrane GJ channels, thus decreasing GJ conductance (Schulz and Heusch, 2004). On the other hand, dephosphorylation of Cx43 by protein phosphatases has been demonstrated to increase single channel conductance in rat cardiomyocytes. Nevertheless, the phosphorylation state of connexins is not reduced to the balance of all these kinases and phosphatases. In fact, kinases and phosphatases may interact between them, thus making regulation by phosphorylation even more complex (Schulz and Heusch, 2004).

4.4.5- Nucleotides

Nucleotides may regulate connexin function rather indirectly by activation of some protein kinases. Thus, cAMP has been shown to activate PKA, therefore phosphorylating Cx43 and increasing GJ intercellular communication. On the other hand, cGMP activates PKG, reducing single channel and cell-to-cell conductance (Kwak and Jongsma, 1996). Moreover, cAMP also induces GJ assembly, increasing Cx43 trafficking to the membrane through PKA-dependent phosphorylation of serine residues located at the carboxy-terminal domain (TenBroek et al., 2001).

Importantly, low ATP levels have been also described to reduce GJ intercellular communication between paired guinea pig cardiomyocytes in a concentration-dependent manner (Sugiura et al., 1990). This effect is probably dependent on a direct interaction between ATP and connexin channels, but not secondary to changes in intracellular concentration of Ca^{2+}, Mg^{2+} and H^{+} (Sugiura et al., 1990). The effects of intracellular ATP levels on junctional channel regulation can be of great importance under ischemic conditions, as during myocardial infarction.

4.5- Connexin pharmacology

As commented before, GJ channels provide the basis for intercellular communication, thus playing an important role in maintaining normal function and development in several tissues. In the cardiovascular system, they allow the existence of synchronized cardiac contraction, but also they participate in the regulation of vascular tone, endothelial function and myoendothelial interaction. Pharmacological modulation of these channels might help to elucidate their role in physiology and pathophysiology, and, in this way, they might become new interesting therapeutic targets (Dhein, 2004). However, the particular location of connexin-formed junctional channels at intercalated disks makes this pharmacological modulation complex. Drugs have to be able to reach this space, and for this reason most GJ modulators are highly lipophilic. However, this carries the handicap that most of these modulators are not specific, and affect other intracellular targets. This problem is now being partially solved with development of specific peptides targeted against some connexin isoforms.

4.5.1- Gap junction inhibitors

Polyalcohols as heptanol or octanol are, without question, the archetypal inhibitor of intercellular communication through GJ. Although their precise mechanisms of action are not completely understood, they have been reported to reversibly block cell-to-cell communication by reducing the open-probability of GJ channels (Bastiaanse et al., 1993). This effect does not seem to be related to a direct action on connexins, but to a modification of the physical properties of cholesterol-rich domains of the plasma membrane, eliciting a conformational change at the interface between GJ proteins and membrane lipids (Bastiaanse et al., 1993). However, effects of polyalcohols are not specific. Heptanol has been also shown to reduce other non-junctional ionic currents, as Na^+ and Ca^{2+} inward currents (Takens-Kwak et al., 1992). Furthermore, some alcohols as ethanol have been shown to activate some PKC isoforms (Inagaki and Mochly-Rosen, 2005). These side effects may hamper a correct interpretation of the findings obtained with those GJ uncouplers.

Other GJ uncouplers have been developed and tested, but their specificity is similar to that of heptanol. Volatile anaesthetics, as halothane or sevofluorane, inhibit intercellular communication, probably by reducing GJ open-channel probability (He and Burt, 2000). However, they also alter non-junctional currents (Terrenoire et al., 2000),

and other cell systems as the sarcoplasmic reticulum (Wheeler et al., 1997). Moreover, the protective effect of pre-treatment with halothane or sevofluorane in rat and rabbit myocardium has been related to adenosine receptors, PKC activation, or mitoK_{ATP} channels (Cope et al., 1997; Coetzee et al., 2000; Bouwman et al., 2006). On the other hand, their protection during myocardial reperfusion may be due to the attenuation of sarcoplasmic reticulum-dependent Ca^{2+} oscillation (Siegmund et al., 1997), but not to their effects on GJ.

Other GJ inhibitors are lipophilic agents, as glycyrrhizic acid derivates, including carbenoxolone or 18α-glycyrrhetinic acid, an aglycone saponine extracted from licorice root, with anti-inflammatory and anti-ulcerous activity, and palmitoleic acid. Although the mechanisms of action of these drugs are not well known, in the case of 18α-glycyrrhetinic acid it seems to involve changes in connexin phosphorylation and GJ assembly, whereas palmitoleic acid might act in a similar way as heptanol. The effects of these drugs require longer incubation times that the previously mentioned. Nevertheless, they have also side effects, with reported actions on Ca^{2+} currents, mRNA synthesis and MPTP opening (Dhein, 1998; Battaglia et al., 2008). Quinidine, mefloquine or retinoic acid, are other examples of GJ uncouplers with low specificity (Juszczak and Swiergiel, 2009).

The recent development of connexin-mimetic peptides has partially solved the problem of the low specificity of GJ uncouplers. They are synthetic oligopeptides, homologous to specific regions of connexin extracellular loops, that bind to unapposed hemichannels, thus avoiding assembly and formation of new intercellular channels. As connexin-mimetic peptides inhibit formation of new GJ channels, but do not interfere with function of existing channels (except for the case of unapposed hemichannels), the time course of their action on cell-to-cell coupling is slow and depends on the turnover rate of GJ. Two of the most commonly used connexin-mimetic peptides are ^{37,43}Gap27 and ^{40,43}Gap26, that are homologous to sequences located at the first and second extracellular loops of Cx43, respectively, (Chaytor et al., 1997). ^{37,43}Gap27 has been shown to block Cx43 hemichannels in a rat glioma cell model (Decrock et al., 2009), whereas ^{40,43}Gap26 has been described to specifically inhibit Cx43 hemichannels in isolated rat hearts (Hawat et al., 2010). However, as happened with all GJ uncouplers, their interaction with other membrane proteins cannot be discarded. In fact, some of these peptides have been shown to also inhibit pannexin hemichannel activity (Dahl, 2007).

4.5.2- Gap junction activators

Enhanced cell-to-cell communication can be afforded by indirect interaction with connexins, as during chronic α and β-adrenergic stimulation, which increases Cx43 expression and thus, GJ intercellular communication (Salameh and Dhein, 2011). However, pharmaceutical interest for GJ modulation has increased since the discovery of a new class of antiarrhythmic peptides, including the antiarrhythmic peptide analogue AAP10 and its derivate rotigaptide (ZP123). Although their mechanism of action is unclear, they may actually increase myocardial GJ conductance rather indirectly, through PKCα-dependent phosphorylation of Cx43 at serine 368, located within the carboxy-terminal domain (Weng et al., 2002). Rotigaptide has been shown to efficiently reduce the incidence of ventricular arrhythmias in an open-chest dog model of myocardial ischemia (Xing et al., 2003; Hennan et al., 2006). These promising findings have led to development of new, smaller and orally bioavailable molecules, as the dipeptide Gap-134 (danegaptide). Gap-134 is also able to reduce the incidence of ventricular arrhythmias in an open-chest dog model of myocardial ischemia (Hennan et al., 2009), and that of atrial fibrillation in rat atrial strips subjected to metabolic stress (Rossman et al., 2009). Moreover, this dipeptide is now undergoing a phase I clinical trial for treatment of atrial fibrillation (Hennan et al., 2009). In addition to their effects on channel permeability, these peptides also increase Cx43 trafficking to cell membrane and enhance GJ assembly (Dhein et al., 2010).

GJ communication can be also maintained by preserving the stability of the open state, without affecting GJ unitary conductance. This is the effect of the peptide RXP-E, which binds to the Cx43 carboxy-terminal domain, partially preventing acidification-induced uncoupling (Lewandowski et al., 2008). Using RXP-E as a reference, new smaller molecules have been designed and analyzed. As a result, ZP2519, having lower molecular weight and higher stability in the cytoplasm, has been recently developed. However, further studies are needed to assess the potential benefits of this oligopeptide in the clinical practice (Verma et al., 2010).

5. Cardiovascular functions of connexins

As mentioned before, GJ channels form low resistance pathways connecting cells, and allowing the transfer of current and the exchange of small molecules between neighbouring cells. The role of GJ in development (Levin, 2002), and in the function of various mature tissues and organs, including the nervous (Bruzzone and Ressot, 1997), immune (Saez et al., 2003; Wong et al., 2004) and digestive systems (Saez et al., 2003), urogenital smooth muscle (Karicheti and Christ, 2001), pancreas (Meda, 1996), endocrine glands (Munari-Silem and Rousset, 1996), and the ear, skin and lens (Gerido and White, 2004) has been the subject of several interesting reviews. Here we are going to mainly focus in the role of connexins in the cardiovascular system, where these channels allow propagation of action potential, and thus, synchronized cardiac contractions. Moreover, they also play a role in regulation of vascular tone, in paracrine signaling and in the control of cell volume.

5.1- Cardiac impulse propagation

Pacemaker activity in the sinoatrial node initiates normal heart beats, which are conducted along the atria and delayed at the atrioventricular node. After that, activation of the entire ventricular myocardium occurs via the specialized conduction system. Under abnormal conditions, pacemaker activity can also be developed in other structures, as the atrioventricular node cells or Purkinje fibers. The process of impulse conduction depends on three main factors, including excitability of single cardiomyocytes, electrical coupling between them, and the network properties of the cardiac tissue (Jansen et al., 2010). Whereas cell excitability is determined by the amount of inward currents I_{Na} and I_{Ca}, electrical coupling is dependent on cell-to-cell communication through GJ (Jalife et al., 1999).

The two main functions of GJ at the sinoatrial node are the maintenance of beating at regular frequency, and the transmission of the impulse to the atrial myocardium (Jansen et al., 2010). However, both functions need only little amount of coupling (Wilders et al., 1996). Immunohistochemical studies have demonstrated that connexin 45 is the predominant isoform at the center of the node, but some controversy exists regarding the presence of other isoforms at the periphery (Boyett et al., 2000). Interestingly, it has been hypothesized that heterotypic GJ, formed by one connexin 45

connexon and one Cx43 connexon, may form at the periphery between sinoatrial and atrial cells, respectively. In this way, these heterotypic GJ would close when current flows from the atria to the node, but would open when current flows from the node to the atria (Jongsma, 2000).

The main connexin isoforms expressed in the atria are Cx43 and connexin 40 (Jansen et al., 2010). Several studies have suggested that connexin 40 deficiency has important effects on atrial conduction. Homozygous connexin 40 knock-out mice have been shown to have reduced atrial conduction velocity. This finding is associated with prolongation of P wave duration in the electrocardiogram (ECG), which reflects total atrial activation time. Moreover, connexin 40 knock-out mice are more vulnerable to supraventricular arrhythmias (Verheule et al., 1999). On the other hand, conditional knock-out mice for Cx43 did not show any effect in P wave duration or atrial conduction velocity, suggesting that Cx43 is not the predominant connexin for atrial conduction in the presence of connexin 40 (Thomas et al., 1998). Importantly, a common finding in human atrial fibrillation (and also in animal models of this common supraventricular arrhythmia) is the abnormal expression of connexin 40 (Nattel et al., 2007). Such abnormal expression levels and distribution may lead to heterogeneous electrical coupling and dispersion of conduction, which is the substrate for atrial arrhythmias. The importance given to GJ in atrial fibrillation is illustrated by the existence of a phase I clinical trial with the GJ opener Gap-134, that is currently being conducted (Hennan et al., 2009).

Three connexin isoforms have been described in the atrioventricular node of mice, connexin 40, 30.2 and 45 (Jansen et al., 2010). The absence of connexin 40 was consistently associated with abnormal atrioventricular conduction, as seen by prolonged PQ intervals in ECG recording (Verheule et al., 1999). On the contrary, heterozygous deficiency for connexin 45 did not modify atrioventricular conduction, although full deletion was not investigated due to its lethality (Kruger et al., 2006). However, in the absence of Cx40, Cx45 haplodeficiency further delays atrioventricular conduction.

Cx43 is the main connexin isoform expressed in the working ventricular myocardium. Cx43 knock-out ($Cx43^{-/-}$) mice die soon after birth, due to cardiac malformations at the pulmonary outflow tract (Reaume et al., 1995). For this reason, studies on the role for Cx43 on ventricular conduction have been performed in heterozygous Cx43 knock-out ($Cx43^{+/-}$) mice (expressing half of the normal content of the protein) or in conditional knock-out mice. The general conclusion obtained from

these studies is that ventricular conduction is affected only when Cx43 is markedly reduced (van Rijen et al., 2004; Danik et al., 2004; Stein et al., 2009). In fact, optical mapping studies have shown that $Cx43^{+/-}$ mice are devoid of changes in conduction, despite having 50% of normal Cx43 content (Morley et al., 1999). It has been suggested that the lack of effect of moderate reductions in GJ communication on impulse propagation are due to the fact that ventricular conduction has a high safety factor, and that more than a 90% reduction in the number of GJ is necessary to induce noticeable effects in impulse propagation (Jongsma and Wilders, 2000). At these low levels, Cx43 expression has been shown to become heterogeneous, increasing anisotropy (Gutstein et al., 2001a; van Rijen et al., 2004). Importantly, a reduction and re-distribution of Cx43 outside the intercalated disks has been described in diseased myocardium. These changes have been associated with an increased incidence of ventricular tachyarrhythmias (Saffitz et al., 2007; Severs et al., 2008). Furthermore, different transgenic mice models of Cx43 deficiency have shown an increased incidence of PVBs and spontaneous and inducible ventricular tachyarrhythmias under baseline conditions (Lerner et al., 2000; Vaidya et al., 2001; Gutstein et al., 2001a; Gutstein et al., 2001b; van Rijen et al., 2004; Danik et al., 2004; Danik et al., 2008). However, the effects of a reduction in GJ communication on arrhythmogenesis under ischemia or reperfusion are not well known, as is if there are differences between a reduction in the number of available GJ channels and a reduction in the unitary conductance of these channels.

5.2- Regulation of vascular tone

Modulation of vascular tone is crucial to maintain circulatory homeostasis. Although neuronal innervation and electrical excitability play essential roles in initiating and propagating vasoactive stimuli among vascular smooth muscle cells, intercellular communication through GJ is responsible for coordinated vessel tone. Cell-to-cell communication through GJ exists not only between smooth muscle cells, but also between endothelial cells, and between endothelial and smooth muscle cells (Christ et al., 1996). Several connexin isoforms have been described to be expressed in both smooth muscle cells and in endothelial cells, including Cx43, connexin 40, 45 and 37, although connexin 37 seems to be more abundant in endothelial cells, and connexin 45 in smooth muscle cells (Christ et al., 1996; Figueroa and Duling, 2009). Connexin expression has been shown to be altered under pathological conditions associated with

vascular complications, as hypertension or diabetes, highlighting the importance of cell-to-cell communication for vascular homeostasis (Figueroa and Duling, 2009).

GJ-mediated electrical coupling between smooth muscle cells has been described to allow spreading of relatively small depolarizations in isolated vascular tissues (Hirst and Edwards, 1989; Holman et al., 1990; Tomita, 1990). The physiological relevance of passive current propagation remains to be demonstrated *in vivo*, although hyperpolarizations and depolarizations have been correlated with vascular tissue relaxations and contractions, respectively, *in vitro* (Beny and Pacicca, 1994; Xia and Duling, 1995). In fact, previous studies suggested that intercellular communication through GJ mediated the vasodilation and vasoconstriction responses observed along the arteriolar wall in resistance vessels (Segal and Duling, 1986). Therefore, triggered vasodilatory responses can be conducted through GJ increasing blood flow into the stimulated vessel (Segal, 1994). In this sense, studies performed in rat aortic rings have shown that pre-treatment with heptanol reduces the rate and magnitude of α1-adrenergic contractions, without any detectable non-junctional effects (Christ et al., 1993).

In addition to these electrophysiological findings, metabolic coupling has been also described in the vascular wall. Diffusion of ions and second messengers, as Ca^{2+} and IP3, has been shown between coupled human vascular smooth muscle cells in culture (Christ et al., 1992). These studies suggested that GJ play an essential role coordinating changes in membrane potential and intracellular Ca^{2+} between adjacent smooth muscle cells (Christ et al., 1992; Christ et al., 1996), a hypothesis later confirmed by its blockade using GJ uncouplers, as ^{37,43}Gap27 and 18α-glycyrrhetinic acid (Earley et al., 2004). Moreover, the extent of metabolic coupling can be modified by vasomodulators, as serotonin (Moore et al., 1991).

Finally, as mentioned before, metabolic and electrical coupling has been also described to exist between smooth muscle cells and endothelial cells. This heterocellular communication seems to play an essential role in the Ca^{2+}-mediated responses induced by endothelium-dependent vasodilators, as acetylcholine (ACh), specifically in small vessels (Figueroa and Duling, 2009). Thus, the relaxant pathway associated with the hyperpolarization of the underlying smooth muscle cells has been attributed to the release and transfer through myoendothelial GJ of an endothelium-derived hyperpolarizing factor (EDHF), whose identity remains still uncertain (Vanhoutte, 2004).

5.3- Paracrine function of connexin hemichannels

The contribution of connexin hemichannels to cell communication remains poorly understood, and available information is mainly restricted to neuronal, glial or endothelial cells. Opening of connexin hemichannels may induce the release to the extracellular space of intracellular metabolites, like ATP or nicotinamide adenine dinucleotide (NAD^+) (Kang et al., 2008; Saez et al., 2010), which could be involved in paracrine signaling. This mechanism may explain propagation of Ca^{2+} waves in astrocytes (Suadicani et al., 2004) and epithelial cells (Frame and de Feijter, 1997), in which several stimuli increase intracellular Ca^{2+} concentrations that propagate from cell-to-cell as a wave. In astrocytes and osteocytes, Ca^{2+} wave propagation depends, at least in part, on the release of ATP to the extracellular space (Guthrie et al., 1999; Cotrina et al., 2000; Jorgensen et al., 2002). ATP would then act on purinergic receptors located in neighbouring cells, thus stimulating IP3 production, and inducing a secondary increase in intracellular Ca^{2+} (Goodenough and Paul, 2003). The involvement of connexin hemichannels on ATP release to the extracellular space and Ca^{2+} wave propagation was demonstrated by the fact that both phenomena were blocked by ^{40,43}Gap26, without modifying GJ intercellular communication (Braet et al., 2003b). Altogether, these data support a role for connexin hemichannels in ATP release to the extracellular space and in receptor-mediated paracrine signaling.

Connexin hemichannels may also modulate cell communication, including cell death, by Ca^{2+}-independent mechanisms. These mechanisms may involve metabolites that can be released from opened connexin hemichannels, as NAD^+ (Bruzzone et al., 2001) and glutamate (Ye et al., 2003; Jiang et al., 2011). In this sense, it has been described that uncontrolled release of glutamate can trigger excitotoxic necrosis or apoptosis of neurons (Zipfel et al., 2000). Moreover, the massive release of ATP through opened hemichannels (Cotrina et al., 1998; Guthrie et al., 1999; Jorgensen et al., 2000; Jorgensen et al., 2002), might also induce apoptosis either through activation of the pore-forming $P2X_7$ purinergic receptors (Tsukimoto et al., 2005), or through the rapid accumulation of adenosine, which causes apoptosis in a neuroblastoma-derived cell line (Schrier et al., 2002).

However, recent findings suggest that the release of metabolites through connexin hemichannels may behave as a double-edged sword. As seen before, a sustained release of these metabolites may cause toxic effects, whereas a transient

opening of hemichannels and release of mediators may participate in protective signaling (Lin et al., 2008; Schock et al., 2008). However, this possible role for connexin hemichannels is, at present, poorly understood.

5.4- Volume regulation

Intracellular edema or cell swelling is often associated with situations of cell injury, as occurs during ischemia-reperfusion (Garcia-Dorado et al., 1993; Saeed et al., 2010). Although mechanisms leading to cell swelling are not completely understood (Garcia-Dorado et al., 2012), opening of connexin hemichannels might allow water influx into cells, therefore inducing intracellular edema. As previously mentioned, the accumulation of end-products of anaerobic metabolism during ischemia, and the rapid wash-out of the extracellular space at the beginning of reperfusion, originates a high water influx, inducing cell swelling (Piper et al., 1998). Several studies have supported a role for connexin hemichannels in volume regulation. First, experiments performed in Xenopus oocytes overexpressing connexin 46 showed permeabilization to Lucifer Yellow and lysis after exposure to low Ca^{2+} concentrations, a condition known to open connexin hemichannels (Paul et al., 1991; Saez et al., 2005). That these effects were secondary to water uptake was suggested by the fact that they were prevented by increasing the osmotic strength of the extracellular buffer with Ficoll (Paul et al., 1991). Later, other studies demonstrated, in a variety of cell types expressing connexins, that a reduction in extracellular Ca^{2+} was associated with significant and reversible increases in volume, an effect that was inhibited by different GJ uncouplers (Quist et al., 2000).

5.5- Functions of mitochondrial Connexin 43

Recent studies have described the presence of Cx43 in mitochondria from rat, mouse, pig and human cardiomyocytes (Boengler et al., 2005; Rodriguez-Sinovas et al., 2006a). Subfractionation studies have shown that Cx43 is mainly located at the inner mitochondrial membrane, where it translocates using the TOM-TIM system, in a Hsp-90-dependent manner (Rodriguez-Sinovas et al., 2006a). Moreover, recent studies have reported that Cx43 is only present in subsarcolemmal, but not interfibrillar, cardiomyocyte mitochondria (Boengler et al., 2009). However, and despite the intense research efforts made, little is known about its physiological function. It has been suggested that mitochondrial Cx43 may form hemichannels at the inner mitochondrial

membrane, and that may control mitochondrial potassium fluxes in cardiomyocytes (Miro-Casas et al., 2009) and astrocytes (Kozoriz et al., 2010). Interestingly, and as will be discussed later, mitochondrial Cx43 might be involved in preconditioning protection (Rodriguez-Sinovas et al., 2006a).

6. Role of Connexin 43 in myocardial ischemia-reperfusion injury

In addition to their physiological functions, connexins, and specially Cx43, have been suggested to be involved also in pathological processes. The role played by intercellular communication through connexin gap junctional channels in spreading of cell death has been solidly demonstrated in myocardium and other tissues. The first evidences suggesting that GJ mediate propagation of myocardial ischemia-reperfusion injury come from studies using computer simulations. These works demonstrated that some kind of interaction between cells was necessary to explain myocardial infarction geometry (Garcia-Dorado et al., 1989). Dead, hypercontracted, cardiomyocytes are not found scattered across the area at risk. Instead, they are connected to other dead myocytes, generating what is known as areas of contraction band necrosis. This pattern could be explained only by the existence of some interaction between adjacent cells (Garcia-Dorado et al., 1989; Garcia-Dorado and Ruiz-Meana, 2000). This interaction was later reported to occur during reperfusion, and to be, at least in part, chemical and mediated through GJ, because the GJ uncoupler heptanol, given after ischemia, was able to lessen the appearance of these areas of contraction band necrosis (Garcia-Dorado et al., 1997). Moreover, heptanol also reduced spreading of hypercontracture between adjacent cardiomyocytes and, when administered at the onset of reperfusion, cell death in isolated rat hearts, and infarct size in *in situ* pig hearts (Garcia-Dorado et al., 1997). These effects were later confirmed by other, chemically unrelated, GJ uncouplers, as 18α-glycyrrhetinic acid, halothane and palmitoleic acid, and it was associated with a slower and attenuated recovery of myocardial electrical resistivity during reperfusion (Rodriguez-Sinovas et al., 2004). This last finding is of great importance, as tissue electrical resistivity depends, among other factors, on GJ communication, and thus, an attenuated recovery during reperfusion in the presence of GJ uncouplers would confirm that these drugs are, in fact, closing GJ channels. Although the mechanisms by which GJ play a role in spreading of hypercontracture and cell death have not been completely elucidated, it has been suggested that it is mediated by passage of Na^+ from injured to healthy cells, and subsequent exchange by Ca^{2+} through the reverse mode of the NCE (Ruiz-Meana et al., 1999). The role for GJ-mediated intercellular communication on propagation of cell death has been observed, thereafter, in other tissues under a variety

of insults. Thus, it has been reported that GJ coupling may mediate spreading of cell injury during cerebral ischemia (Lin et al., 1998; Rami et al., 2001), and may also enhance neuronal damage during brain traumatic injury (Frantseva et al., 2002).

In addition to the involvement of Cx43-mediated intercellular communication through GJ in cell death during myocadial reperfusion, spreading of cell damage during ischemia, in the absence of flow restoration, has been also suggested. Contrary to the general belief that ischemia causes GJ-channel closure, studies in intact rat myocardium have provided direct evidences that they remain partially opened during the first minutes of ischemia (Ruiz-Meana et al., 2001), allowing passage of many pathophysiologically relevant molecules. This may result in synchronization and/or propagation of cytosolic derangements during ischemia, leading to synchronization of rigor contracture, and spreading of cell death (Ruiz-Meana et al., 2001). Confirming these data, it has been described that pre-treatment with heptanol just before the ischemic period, reduces infarct size in isolated rabbit hearts (Saltman et al., 2002). Similar findings have been observed with several GJ uncouplers given during hypoxia in isolated rat hearts (Rodriguez-Sinovas et al., 2006b). Moreover, studies performed in $Cx43^{+/-}$, having about 50% of normal Cx43 content, submitted to coronary occlusion without reperfusion, have shown reduced infarct size (Kanno et al., 2003), although this effect was not confirmed later in the same model after reperfusion by other authors (Schwanke et al., 2002).

On the other hand, connexin hemichannels have been described to open transiently during simulated ischemia in rat neonatal cardiomyocytes. In this way, they may potentially contribute to intracellular Ca^{2+} overload and cell swelling, and promote cell injury during simulated reperfusion, as suggested by the fact that blockade with the hemichannel inhibitor ^{40,43}Gap26 improved cell viability (Shintani-Ishida et al., 2007). These findings led some authors to suggest that opened connexin hemichannels may compromise ionic homeostasis during ischemia, promoting ventricular arrhythmias and myocardial injury (John et al., 1999; Kondo et al., 2000; John et al., 2003). This hypothesis has received further support by the recent finding that treatment with ^{40,43}Gap26, either before or after ischemia, reduced infarct size in isolated, Langendorff-perfused, rat hearts submitted to global or regional ischemia and reperfusion (Hawat et al., 2010; Johansen et al., 2011). However, some of these findings have not been confirmed by other authors (Clarke et al., 2009), leaving the role of Cx43 hemichannels in ischemia-reperfusion injury an unsolved question.

Despite the previously mentioned evidences supporting a role of Cx43, either as gap junctional channels or hemichannels, in ischemia-reperfusion injury, other studies have shown conflicting results. Treatment with the antiarrhythmic peptides rotigaptide and Gap-134, which have been shown to improve GJ communication and reduce the incidence of ventricular arrhythmias, decreases infarct size after regional ischemia-reperfusion in anaesthetized rats (Haugan et al., 2006) and dogs (Hennan et al., 2006; Hennan et al., 2009). Differences between these studies and the previous ones are not clear, but it has been suggested that depending on the severity of injury, GJ may have positive effects, spreading survival agents or diluting cytosolic changes induced by different manoeuvres (Yasui et al., 2000; Garcia-Dorado et al., 2004). Moreover, pharmacological agents used in these and previous studies are not selective, and their actions may depend, in fact, on side-effects of these drugs, independent of GJ uncoupling. One of the aims of this doctoral thesis is to clarify the role of Cx43 in ischemia-reperfusion injury, using transgenic mice models of Cx43 deficiency. As complete ablation of Cx43 is incompatible with life (Reaume et al., 1995), two different approaches have been used: a knock-in mice model (Cx43KI32), in which Cx43 has been replaced by Cx32, a hepatic isoform with lower conductivity and permeability, and a conditional knock-out model ($Cx43^{Cre\text{-}ER(T)/fl}$), in which a marked reduction of Cx43 expression is achieved after administration of 4-hydroxytamoxifen (4-OHT).

7. Role of Connexin 43 in preconditioning protection

As commented before, despite the huge research efforts made in this field, the end-effectors by which IPC can prevent cell death secondary to prolonged ischemia and reperfusion are not completely understood. It was initially suggested that one of these end-effectors could be the restriction of GJ-mediated propagation of injury (Garcia-Dorado et al., 2002; Miura et al., 2004). This hypothesis was based in the fact that PKCε and MAPK, two of the most prominent kinases involved in the preconditioning cascade, are also known to regulate Cx43 phosphorylation and function (Lampe and Lau, 2000; Cruciani and Mikalsen, 2002). Furthermore, preconditioned hearts have been described to retain higher Cx43 levels as compared to non-preconditioned hearts (Daleau et al., 2001), and to preserve Cx43 phosphorylation during the following prolonged ischemia (Schulz et al., 2003). Further supporting an involvement of Cx43-formed GJ in preconditioning protection, it was shown that administration of the GJ blocker heptanol before preconditioning, abolished its protective effect in isolated mice hearts (Li et al., 2002a).

However, detailed electrophysiological analyses showed minimal or no effect of previous IPC on active (action potential characteristics and propagation) or passive myocardial electrical properties (electrical impedance) during prolonged ischemia (Padilla et al., 2003). More importantly, preconditioning had no effect on the rate of normalization of electrical coupling during reperfusion (Padilla et al., 2003). These results argued strongly against any contribution of attenuated GJ-mediated spreading of cell death in the protective effect of preconditioning, and suggest that the relation between Cx43 and preconditioning should be independent of cell-to-cell communication through GJ.

While the contribution of GJ as end-effectors of preconditioning protection remains elusive, very solid evidence on the involvement of Cx43 is becoming available. The most conclusive support for a role of Cx43 in IPC has been provided by studies showing that Cx43 deficiency in $Cx43^{+/-}$ mice completely abolished preconditioning protection (Schwanke et al., 2002; Schwanke et al., 2003). Interestingly, isolated cardiomyocytes from these mice were also unable to be preconditioned (Li et al., 2004). As isolated cardiomyocytes are not able to form GJ, abolition of protection by Cx43 deficiency could not be explained by changes in cell-to-cell communication (Li et al.,

2004), as previously suggested by electrical impedance measurements (Padilla et al., 2003).

One possibility that could explain the GJ-independent effects of Cx43 in preconditioning protection would be a contribution of unapposed hemichannels. Several data indicate that connexin hemichannels may behave as a double-edged sword. Whereas a sustained opening and release of metabolites, as ATP, may cause toxic effects through receptor-dependent or independent mechanisms (Schrier et al., 2002; Braet et al., 2003a; Braet et al., 2003b; Tsukimoto et al., 2005), a transient opening may participate in protective signaling (Lin et al., 2008; Schock et al., 2008). Lin et al. demonstrated that glial C6 cells devoid of Cx43 were insensitive to preconditioning, whereas protection in C6 cells overexpressing Cx43 was attenuated by small interference RNA raised against Cx43, or by connexin channel blockers. Furthermore, the same authors demonstrated that preconditioning protection was linked to a marked increase in the amount of Cx43 hemichannels, and mediated by adenosine (Lin et al., 2008). From these results, other authors suggested that efflux of ATP through connexin hemichannels led to the extracellular accumulation of its catabolic product, adenosine, which has a potent protective effect through interactions with its specific membrane receptors (Laubach et al., 2011). However several authors have not been able to prove a role of Cx43 hemichannels in myocardial preconditioning (Vessey et al., 2010; Vessey et al., 2011). Anyway, the involvement of Cx43 connexons in preconditioning protection against ischemic injury is poorly understood, and other authors have suggested that preconditioning may, in fact, inhibit opening of connexin hemichannels during index ischemia, thus reducing cell death through a decrease in the cytoplasmic depletion of metabolites and in the harmful release of intracellular messengers (Miura et al., 2010a).

A breakthrough in the elucidation of the role of Cx43 in preconditioning may have been recently achieved with the description of the presence of Cx43 in the mitochondria of cardiomyocytes from mice, rat, pig and humans (Boengler et al., 2005; Rodriguez-Sinovas et al., 2006a). This organelle is particularly important during ischemia-reperfusion and IPC. As commented before, MPTP opening and ROS production by mitochondria contribute to cell death, and its modulation by preconditioning might contribute to myocardial protection. Thus, ROS have been described to be involved in ischemia-reperfusion injury (Lefer and Granger, 2000), and on the other hand, small amounts of ROS seem to be mediators of IPC protection

(Schulz et al., 2007). In this context, Cx43 localization in the mitochondria seems to be very important. Thus, Cx43 deficiency in cardiomyocytes from $Cx43^{+/-}$ mice has been associated with a decrease in ROS formation after treatment specifically with Dzx, a drug supposed to induce pharmacological preconditioning through actions on mitoK_{ATP} channels (Heinzel et al., 2005). This effect was linked to loss of Dzx protection in these mice (Heinzel et al., 2005). Furthermore, inhibition of Cx43 translocation to the mitochondria, without modifying total Cx43 content, abolished Dzx protection in isolated rat hearts (Rodriguez-Sinovas et al., 2006a). Altogether, these data suggest that Cx43 play an important role in the preconditioning signaling mediated by ROS. The importance of Cx43 in mitochondrial function is highlighted by recent studies demonstrating that mitochondrial Cx43 modulates K^+ influx into the mitochondria under different conditions (Miro-Casas et al., 2009). However, metabolic consequences of Cx43 deficiency, probably related to its absence in the mitochondria, are not known, as is whether the role of Cx43 in ischemic and pharmacological preconditioning is shared by other connexin isoforms. Thus, we will use a Cx43KI32 mice model to assess both issues and a conditional knock-out model ($Cx43^{Cre\text{-}ER(T)/fl}$), to discard that the presence of Cx32 could contribute to these effects.

8. Unsolved questions regarding the role of Connexin 43 in ischemia-reperfusion injury and preconditioning protection

As commented before, most of the previous studies have analyzed the role of Cx43 in ischemia-reperfusion injury using pharmacological agents with low selectivity and lots of side-effects. Furthermore, studies in $Cx43^{+/-}$ mice have obtained conflicting results (Schwanke et al., 2002; Kanno et al., 2003). Moreover, metabolic effects of Cx43 are unknown, as is whether the role of Cx43 in ischemic and pharmacological preconditioning is shared by other connexin isoforms. Thus, the aims of this doctoral thesis are to assess the role of Cx43 in myocardial metabolism, in ischemia-reperfusion injury, and in preconditioning protection. For these purposes two different transgenic mice models of Cx43 deficiency have been used. The Cx43KI32 mice model, in which Cx43 has been replaced by Cx32, a hepatic isoform with lower conductivity and permeability, and the $Cx43^{Cre\text{-}ER(T)/fl}$ model, in which a marked reduction of Cx43 expression is achieved after administration of 4-OHT.

These two models would represent two different systems of reducing GJ communication. The first one would represent a reduction in unitary conductance of connexin channels, whereas the second one would correspond to an extreme reduction in the number of available connexin channels. Developing drugs able to modulate connexin conductance could allow translation of these strategies to patients. However, effects of these treatments on arrhythmogenesis must be first considered, because, as mentioned before, GJ uncoupling may generate arrhythmias. The effects of both maneuvers on ventricular arrhythmias during ischemia-reperfusion have not been previously analyzed.

Hypothesis and aims

1. Hypothesis

Connexin 43 deficiency, either by its replacement by connexin 32 (i.e., a reduction in maximal unitary conductance of connexin channels), or by inducing a marked decrease in its expression (i.e., a marked reduction in the number of available connexin channels) in transgenic mice, would modify myocardial energetic metabolism, alter myocardial tolerance to ischemia-reperfusion injury, and change susceptibility to ischemic and/or pharmacological preconditioning. These effects will be probably associated with changes in the incidence of ventricular arrhythmias during ischemia-reperfusion.

2. Aims

To analyze the effects of genetic connexin 43 deficiency, either by reducing maximal unitary conductance or the number of available connexin channels, on:

1. Cardiac energetic metabolism.
2. Myocardial tolerance to ischemia-reperfusion injury.
3. Susceptibility to ischemic and pharmacological preconditioning.
4. The incidence of ventricular arrhythmias, and myocardial electrophysiological properties.

Material and methods

1. Chemical reagents and drugs

The Laemmli buffer 2x, the Electrophoresis buffer 10x and the Acrylamide/Bis-acrylamide 37.5:1 were obtained from BioRad (United States).

The Superblock Blocking Buffer and the SuperSignal West Dura Extended Duration Chemiluminescent Substrate were purchased from Thermo Scientific-Pierce Protein Research Products (United States).

The Wizard SV Genomic DNA Purification System for polymerase chain reaction (PCR) was obtained from Promega (United States).

The chemical reagents used for Master Mix preparation for PCR analysis, as well as the primers, the SYBRsafe and the Cyano/Orange buffer, were purchased from Invitrogen (United States).

The Agarose type I, Molecular Biology Grade, was obtained from Calbiochem (United States).

All other chemical reagents included in the buffers used in experiments in isolated mice hearts and cadiomyocytes, together with ethanol, methanol, isopentane, 2,3,5-triphenyltetrazolium chloride (TTC), reduced nicotinamide adenine dinucleotide (NADH), sodium pyruvate, fetal bovine serum, paraformaldehyde, Triton X-100, $NaBH_4$, sodium azide, glycine, sodium fluoride, sodium orthovanadate (Na_3VO_4), protease inhibitor cocktail, EDTA, Tris-HCl, NaOH, Tween 20, SDS, ammonium persulfate, TEMED, Ponceau S, PBS, EGTA, MOPS, PMSF, proteinase-k, para-aminobenzoic acid (PABA), chloroform, 3-(trimethylsilyl)propionic acid-d4 sodium salt (TSP), Dzx, cariporide, and 4-OHT were obtained from Sigma-Aldrich Chemie (Germany).

2. Animals

All the studies were approved by the Ethical Committee of the Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, and were performed in accordance with the European Union legislation. Two transgenic mice models of Cx43 deficiency were used, both with the same genetic background: mixed 129P2/OlaHsd-C57BL6 (Plum et al., 2000; van Rijen et al., 2004; Eckardt et al., 2004). Homozygous Cx43KI32 mice have a reduction in maximal unitary conductance of connexin channels due to the replacement of the coding region of Cx43 by that of Cx32. In contrast, $Cx43^{Cre/fl}$ from the conditional knock-out $Cx43^{Cre\text{-}ER(T)/fl}$ strain express about 50% of normal Cx43 content under baseline conditions, whereas after treatment with 4-OHT, Cx43 expression is markedly reduced, therefore representing a strong reduction in the number of available connexin channels.

Animals were bred and maintained with water and food *ad libitum,* in maintenance rooms of the Animal Facilities of the Vall d'Hebron Research Institute, without further conditions than those considered standard by the Institution.

2.1- Cx43KI32 mice

Cx43KI32 mice were obtained from the European Mouse Mutant Archive (EMMA). These animals, in which the coding region of the Cx43 gene is replaced by that of Cx32, were developed by Plum et al. (Plum et al., 2000), using a double replacement strategy. Heterozygous (HET) mutants are fertile and co-express the wild type and the Cx32 allele (Cx43/Cx32), whereas homozygous (HOM) mutant mice express only Cx32 (Cx32/Cx32), and homozygous wild-type (WT) mice express only Cx43 (Cx43/Cx43). PCR analysis of DNA extracted from tail samples was used to confirm genetic substitution of Cx43 in HET and HOM mutants, and immunohistological examination was performed to assess the distribution of each connexin isoform.

2.1.1- PCR analysis

Genetic substitution of Cx43 by Cx32 was confirmed by PCR analysis of DNA extracted from tail samples of all experimental animals. DNA was extracted overnight, at 55ºC, using a mix solution containing Proteinase-k (20 mg/ml), Nucleic lysis

solution, EDTA (0.5 M, pH 8), and RNAse A (4 mg/ml), all included in the Wizard SV Genomic DNA Purification System (Promega). Thereafter, 250 µl of the Lysis buffer from the same manufacturer were added to the samples, and all the content was rapidly transferred into a minicolumn with a collection tube. To purify DNA, each sample was centrifuged four times at 13000 g (5403 Eppendorf centrifugue, Germany), at room temperature (RT). DNA concentration was measured in a NanoDrop system (Thermo Scientific, United States). Samples were diluted when required to reach a working concentration of 10 ng/µl.

Four different primers (Invitrogen, United States) were used for DNA amplification, as previously described (Plum et al., 2000): Cx43-Forward (5'-cctcctgggtacaagctgg-3') and Cx43-Reverse (5'-actcactcatgtatacagaacc-3') for Cx43-DNA sequences, and Cx32-HO2 (5'-cgtcttcactgtctttatgctcgc-3') and Cx43-RO4 (5'-cgcctcattactgaggttgttgag-3') for DNA sequences coding for Cx32. Two Master Mix solutions were made, containing a deoxynucleotide Mix (dNTPs, 10 mM), Platinum Taq Polymerase (5 u/µl) and primers (4 µM) corresponding to Cx43 or Cx32, in a solution of Taq buffer 10x, $MgCl_2$ (50 mM), and sterile distilled water. Five µl of the samples containing the purified DNA were added to 20 µl of the Master Mix buffer, and the final solution was incubated in a thermocycler (Thermal i-Cycler, BioRad). The conditions for amplification were: one cycle of first denaturalization at 95ºC for three minutes, and 35 cycles of denaturalization at 95ºC for 20 seconds, alignment at 58ºC during 45 seconds and synthesis at 72ºC, for one minute. This was followed by a final synthesis cycle at 72ºC during three minutes.

The end-product of PCR was then mixed with Cyano/Orange buffer (1%), and loaded into the wells of an agarose gel (1.5% in TBE 1x) containing 10% of SYBRsafe. Electrophoresis was performed by application of a current of 90 V for 15 min (Power Pac 300, BioRad). Fluorescent signals corresponding to amplified DNA sequences were observed by UV-transilumination in a Molecular Imager Gel Doc XR System (BioRad), and captured using the software Quantity One (BioRad).

2.1.2- Immunohistology

Distribution of Cx43 and Cx32 in WT, HET and HOM hearts was assessed by immunohistology. Hearts were frozen in isopentane at -80ºC. Cryosections of 6 µm were obtained using a cryostat (CM3050 S, Leica, Germany), and transferred to a

microscope slide. Cryosections were fixed in paraformaldehyde (4%) and incubated for 10 minutes with permeabilization buffer (Triton X-100 (0.5%), sodium azide (0.02%) and glycine (100 mM) in PBS). Non-specific binding was, then, blocked with permeabilization buffer containing 5% of fetal bovine serum. Cryosections were incubated for one hour at RT with a rabbit polyclonal antibody raised against Cx43 (71-0700; Zymed, Germany; dilution 1:50) or a rabbit polyclonal anti-Cx32 antibody (C3595, Sigma, United States, dilution 1:100). Once incubation with connexin antibodies finished, cryosections were washed in PBS containing glycine 100 mM, and incubated again, for one hour (RT), with a mouse monoclonal antibody raised against pan-cadherin (C1821, Sigma, Germany, dilution 1:500), to identify intercalated disks. Microscope slides were, then, washed and incubated with the secondary antibodies Alexa Fluor 594 anti-rabbit and Alexa Fluor 488 anti-mouse (Molecular Probes, United States, dilution 1:200) for 60 minutes. Nuclei were stained with Hoeschst 33342 (10 μg/ml). As negative control, samples were not incubated with primary antibodies. Presence and distribution of Cx43 and Cx32 were analyzed by confocal laser scan microscopy (FluoView-1000 confocal microscope, Olympus, Germany).

2.2- $Cx43^{Cre\text{-}ER(T)/fl}$ mice

This conditional model, which avoids the developmental compensation of Cx43 deficiency, is based on a fusion construct of the Cre recombinase and a specifically mutated version of the ligand-binding domain of the human estrogen receptor (ER(T)). Treatment with 4-OHT leads to binding of the drug to the ER(T) domain, inducing Cre activity, whereas ER(T) is insensitive to the natural ligand, β-estradiol. Cre-ER(T) was targeted into the endogenous Cx43 locus to direct deletion to cells expressing Cx43 (Eckardt et al., 2004), thus creating $Cx43^{Cre/fl}$ mice, which express about 50% of normal Cx43 content. $Cx43^{Cre/fl}$ mice, and their corresponding genetic controls $Cx43^{fl/fl}$, were injected intraperitoneally once per day for five consecutive days with 3 mg 4-OHT suspended in plant oil, or vehicle, as previously described (van Rijen et al., 2004; Eckardt et al., 2004). Therefore, four experimental groups were considered: two groups in which Cx43 expression was normal, corresponding to $Cx43^{fl/fl}$ mice either treated with plant oil or 4-OHT, and $Cx43^{Cre/fl}$ animals, that when treated with vehicle have about 50% of normal Cx43 expression, but after treatment with 4-OHT show very low amounts of Cx43, below 10% of normal content. The $Cx43^{fl/fl}$ group treated with 4-

OHT served as a control of possible side effects of the drug. All animals were included in the experiments 14 days after first induction, when Cx43 expression was reduced to those levels.

2.2.1- PCR analysis

Genotype was confirmed by PCR analysis of DNA extracted from ear samples of all experimental animals. The protocol was similar to that described in Cx43KI32 mice, but with some modifications. In this strain, DNA extraction was made according to the Hot Shot method (Truett et al., 2000). An alkaline buffer containing NaOH (20 mM) and EDTA (0.2 mM, pH 12) was added to ear samples and incubated in the thermocycler for 30 minutes at 95ºC, reducing the temperature to 4ºC after this period. The same volume of a neutralization buffer (Tris HCl 40 mM, pH 5) was added to stop the reaction. DNA concentration was, then, measured in the NanoDrop system and samples were diluted when required to reach a working concentration of 10 ng/µl.

Cre DNA sequence was amplified using the following specific primers (Invitrogen, United States): Cre-26 (5'-cctggaaaatgcttctgtccg-3') and Cre-36 (5'-cagggtggtataagcaatccc-3'). The Master Mix solution was prepared using the same conditions than in Cx43KI32 mice, but changing the primers. The conditions for amplification in the thermocycler were: one cycle of first denaturalization at 94ºC for two minutes, and 40 cycles of denaturalization at 94ºC for 45 seconds, alignment at 60ºC during one minute and synthesis at 72ºC, for one minute. This was followed by a final synthesis cycle at 72ºC during 10 minutes. Once PCR end-products were obtained, all remaining steps, including agarose gel preparation, addition of Cyano/Orange buffer (1%) to the Master Mix end-product, loading of samples in gel wells, electrophoresis and DNA detection by fluorescence were performed using the same protocol and conditions than those described for Cx43KI32 mice.

2.2.2- Assessment of Cx43 depletion by western blot

Ablation of Cx43 by 4-OHT injection was confirmed by western blot analysis of total cardiac extracts in all experimental hearts, according to standard procedures (Boengler et al., 2005). Additionally, expression of Cx43 was also assessed by western blot in mitochondrial fractions obtained from 12 mice hearts (3/group), to confirm that Cx43 levels were also reduced in cardiac mitochondria from $Cx43^{Cre/fl}$ mice treated with 4-OHT.

2.2.2.1- Total cardiac extracts

To obtain total cardiac extracts, frozen hearts (lacking both atria and great vessels) were homogenized (Diax 600 homogenizer, Heidolph, Germany), in a homogenization ice-cold buffer containing Tris-HCl (20 mM), NaCl (140 mM), EDTA (0.8 mM, pH 7.8), sodium fluoride (1 mM), sodium orthovanadate (1 mM) and the protease cocktail inhibitor (1%). Nuclei and other tissue debris were discarded by centrifugation at 750 g for 10 minutes (4ºC). A small volume of the supernatant was used to quantify protein concentration according to the Bradford method (BioRad protein assay). The remaining supernatant was mixed with Laemmli buffer 2x (1:1) and frozen at -80ºC.

2.2.2.2- Isolation of mitochondrial ventricular fractions

Fresh cardiac ventricles were minced and homogenized using a potter-teflon at 250 rpm in ice-cold isolation buffer, containing sucrose (290 mM), MOPS (5 mM), EGTA (2 mM, pH 7.4), PMSF (0.5 mM), sodium fluoride (5 mM), sodium orthovanadate (1 mM) and the protease cocktail inhibitor (1%). Mitochondria were, then, isolated by differential centrifugation. First, nuclei and other cell debris were discarded by an initial centrifugation step at 750 g for 5 minutes (4ºC). The supernatant was centrifuged at 5000 g for 3 minutes at 4ºC, and the resulting pellet, containing mitochondria, was resuspended in isolation buffer added with Percoll (17%) (Rodriguez-Sinovas et al., 2006a). Plasma membrane contamination was further removed by an additional centrifugation at 12500 g for 8 minutes at 4ºC, and the resulting pellet was resuspended in isolation buffer and centrifuged again at 5000 g for 3 minutes (4ºC). To increase the degree of purification of mitochondrial fractions, and remove the Percoll buffer, this step was performed three consecutive times. Finally, the pellet was resuspended in 100 µl of isolation buffer and protein concentration was determined by the Bradford method. The remaining volume was mixed with Laemmli buffer 2x (1:1) and frozen at -80ºC.

As a control, and to confirm in these 12 animals that treatment with 4-OHT was effective in reducing total Cx43 levels, expression of the protein was also assessed in brain samples. Total extracts from brain homogenates were obtained using the same procedure than in the case of cardiac extracts.

2.2.2.3- Western blot analysis

After obtaining total cardiac extracts and mitochondrial fractions, Cx43 expression was quantified by western blot according to standard procedures (Miro-Casas et al., 2009). Proteins were separated by their molecular weight through polyacrylamide gel electrophoresis. The running gel (10%) composition was: acrylamide/bis-acrylamide 37.5:1 (10%), Tris base (0.375 mM), SDS (0.1%), ammonium persulfate (0.05%) and TEMED (0.05%), pH 8.8. The stocking gel (4%), that was prepared above the running gel, contained acrylamide/bis-acrylamide 37.5:1 (4%), Tris base (0.125 mM), SDS (0.125%), ammonium persulfate (0.06%) and TEMED (0.125%), pH 6.8.

Electrophoresis gels were assembled in a Mini-Protean III system (BioRad), while immersed in diluted electrophoresis buffer (1x) (BioRad), containing Tris (25 mM), Glycine (192 mM) and SDS (0.1%), pH 8.3. Samples were loaded, together with a protein molecular weight marker (Precision Plus Protein Standards Dual Color, BioRad), into the wells of the system, and a constant current intensity of 40 mA was applied for two hours through a Power Pac 300 (BioRad), to allow separation of the different bands of the molecular marker. Then, proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Sweden) by wet transference, for one hour at a constant voltage of 100 V. Membranes were immersed during transference in an ice-cold transfer buffer, containing methanol (20%), Tris base (25 mM) and glycine (20 mM), maintained under constant shaking. At the end of the transference, membranes were stained with Ponceau S to confirm that protein loading was similar in all samples.

After Ponceau S staining, membranes were washed with PBS and unspecific binding was blocked with PBS containing 1% powdered skimmed milk (PBS-milk) for one hour. Then, they were incubated overnight, in a cold chamber (4ºC), with a monoclonal mouse antibody raised against the amino-terminal tail of Cx43 (Cx43NT1, Fred Hutchinson Cancer Research Center, United States, dilution 1:1000 in PBS-milk 1%). Once incubation with the primary antibody was finished, membranes were washed with PBS-milk 1%, and incubated again, for one hour at RT, with the secondary anti-mouse antibody (A4416, Sigma, France, dilution 1:5000 in PBS-milk 1%). Immunoreactive bands were detected using the SuperSignal West Dura Extended Duration Substrate, and chemioluminiscence was densitometrically quantified (CCD LAS-3000 chamber and Image Gauge analysis software, Fujifilm, Japan).

In order to further confirm a similar protein load in all wells, membranes were later analyzed for expression of a control protein, in this case mitochondrial succinate-ubiquinol oxidoreductase (Oxphos CII). First, membranes were submitted to stripping, to remove binding of previous antibodies, by incubation in a solution of 100 mM glycine (pH 2.5) for 20 minutes. Then, membranes were washed with TBS-T (Tris base 20 mM, NaCl 138 mM, Tween 20 0.1%) and unspecific binding was blocked with TBS-T containing 5% powdered skimmed milk. After blockade, membranes were incubated with a mouse anti-human Oxphos CII antibody (Mitosciences, United States, dilution 1:10000). To exclude sarcolemmal contamination in mitochondrial fractions, membranes were also incubated with a rabbit antibody raised against the α2 subunit of the sarcolemmal Na^+/K^+ ATPase (Upstate Biotechnology, United States, dilution 1:4000). Both Oxphos CII and Na^+/K^+ ATPase antibodies were diluted in TBS-T added with 10% of Superblock Blocking Buffer. After 90 minutes, membranes were washed with TBS-T and incubated again with secondary anti-mouse (Thermo Scientific-Pierce Protein Research Products, dilution 1:1000) or anti-rabbit (Thermo Scientific-Pierce Protein Research Products, dilution 1:1000) antibodies, for detection of signals corresponding to Oxphos CII or Na^+/K^+ ATPase, respectively. Secondary antibodies were diluted in TBS-T containing 5% powdered skimmed milk. Immunoreactive bands were detected using the SuperSignal West Dura Extended Duration Substrate and densitometry analyzed as previously described for Cx43.

2.2.3- Immunohistology

Distribution of Cx43 was assessed by immunohistology in hearts from the four experimental groups, using the same protocol described in point 2.1.2 of material and methods. Cryosections were incubated with a rabbit polyclonal antibody raised against Cx43 (71-0700; Zymed, Germany; dilution 1:50) and a mouse monoclonal antibody raised against pan-cadherin (C1821, Sigma, Germany, dilution 1:500).

3. Isolated mice heart model. Experimental preparation

The experimental model used in all procedures was the isolated, Langendorff-perfused, mice heart preparation, where hearts are retrogradely perfused through the aorta (Bell et al., 2011). This model was considered suitable for the aims of this thesis because it allows a proper physiological study of the heart, but reducing the variability of *in vivo* models. Our cardiac preparation lacks, thus, any influence from hormones or cells present in blood, which is washed by the perfused solution.

Transgenic Cx43KI32 and $Cx43^{Cre\text{-}ER(T)/fl}$ mice of both sexes (14-16 weeks old, the different groups matched to have about 50% males) were submitted to thoracotomy under intraperitoneal anaesthesia with pentobarbital (1.5 g/kg). Whole hearts were quickly excised and perfused retrogradely through the aorta with an oxygenated (95% O_2 : 5% CO_2) Krebs solution at 37ºC, containing, in mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 11, pH 7.4. In this way, the buffer distributes through the coronary arteries, perfusing the whole heart, and allowing cardiomyocytes to survive. Perfusion was performed in a Langendorff apparatus, using the constant flow mode, initially adjusted to produce a perfusion pressure (PP) of 80-90 mmHg under normoxic conditions. A water-filled latex balloon was placed in the left ventricle to monitor left ventricular pressure (LVP). The balloon was inflated to obtain a LVEDP between 6 and 8 mmHg. Left ventricular developed pressure (LVdevP) was calculated as the difference between the systolic peak and LVEDP. PP and LVP were monitored through water-filled pressure transducers (MLT0699 Disposable BP Transducers, ADInstruments, Australia), and signals were amplified and digitized using an analogue-to-digital converter (Powerlab/8SP SP9150, ADInstruments, Australia). Digitized signals were displayed in a computer using the software provided by the supplier (Chart 5.0, ADInstruments), and stored for later analysis.

Hearts were continuously paced by means of two electrodes (Unipolar Temporary Atrial Pacing Lead, Medtronic, United States) placed in the cardiac apex, using rectangular pulses of 2.5 ms duration and 4 V of amplitude, at 133 ms of basic cycle length (BCL, equivalent to 450 bpm). As the Langendorff apparatus was prepared for two simultaneous isolated hearts, the vast majority of experiments were conducted paired (Figure 6).

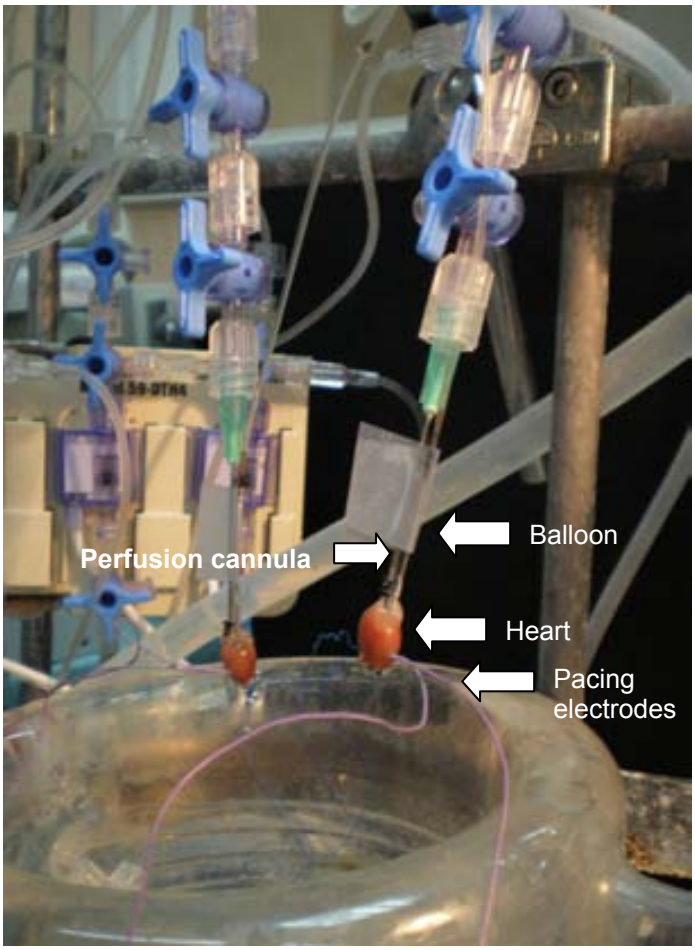


Figure 6.- Langendorff apparatus for isolated, perfused, mice hearts. In the image, it is possible to see two mice hearts cannulated through the aorta, together with the balloon placed in the left ventricle and the pacing electrodes inserted in the cardiac apex.

4. Experimental procedures

4.1- Effects of replacement of Cx43 by Cx32 in Cx43KI32 mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

Cx43KI32 mice, in which Cx43 has been replaced by Cx32, a connexin isoform with lower conductivity and permeability, mainly located in liver, mimic a situation of reduced maximal unitary conductance of connexin channels. This model allows first, to assess the effects of such replacement on energetic metabolism, ischemia-reperfusion injury and cardioprotection, and second to investigate whether these roles of Cx43 are related to specific properties of this connexin isoform.

4.1.1- Effects of replacement of Cx43 by Cx32 on energetic metabolism

Energetic metabolism was analyzed in cardiac extracts and in isolated, Langendorff-perfused, mice hearts by ^{1}H and ^{31}P-NMR spectroscopy, respectively.

4.1.1.1- ^{1}H-NMR spectroscopy

Using this method, we were able to quantify metabolite concentrations in cardiac extracts from WT and HOM animals (n=6 for each genotype), as previously described (Barba et al., 2007). Extracts were prepared from frozen hearts minced in a mortar filled with liquid N_2. Six µl of PABA (100 mM) were added into the mortar as an internal standard of the extraction procedure to control possible tissue losses. Homogenized hearts were immersed in 3 ml of an ice-cold solution of methanol-chloroform 2:1 during 15 minutes. The resulting suspension was then mixed with additional chloroform (1.25 ml) and distilled milli-Q water (1.85 ml), and centrifuged at 1000 g for 5 minutes. As a result, two well-defined phases were obtained with a debris interface in the middle. The aqueous phase (above) and the organic phase (below) were separated and allowed to evaporate at RT under N_2 flow. To minimize metabolite losses, the procedure was repeated again using the interface as departure tissue.

The organic phase was conserved for later analysis, whereas the aqueous phase, containing our metabolites of interest, was dried in a Speed-Vac (vacuum centrifugal evaporator RC10.10, connected to the high vacuum pump RCT.90, Thermo Fisher Scientific-Jouan, United States). The resulting dry-samples were frozen and lyophilized

(lyophilizer LT-105, Christ, Germany). Protein concentration was quantified in the debris interface by the Bradford method (BioRad protein assay).

Lyophilized samples were analyzed by ^{1}H-NMR in a spectrometer (Avance 400, Bruker, Germany). Fully relaxed spectra from tissue extracts were obtained at 9.4 T. The areas of peaks of interest were measured by deconvolution and compared to that of 1 mM of TSP, used as chemical shift standard.

4.1.1.2- ^{31}P-NMR spectroscopy

This method allowed us to analyze phosphocreatine (PCr) and ATP kinetics in isolated hearts from WT and HOM mice (n=4 for each genotype). Cardiac preparations were retrogradely perfused through the aorta with an oxygenated phosphate-free Krebs solution (37ºC). Spectroscopy was performed in an adapted Langendorff apparatus, in which isolated hearts were placed inside the Avance 400 Bruker spectrometer, enabling us to acquire spectra simultaneously to the normoxic perfusion. This procedure had been previously described in rats (Inserte et al., 2009). Spectra were taken during 16 minutes with a delay of 0.6 seconds between pulses, thus resulting in the accumulation of 1500 consecutive scans. PCr and ATP peak areas were measured by integration with the software provided by Bruker (Germany).

4.1.2- Effects of replacement of Cx43 by Cx32 on myocardial tolerance to ischemia-reperfusion injury

Isolated hearts from WT (n=22), HET (n=16) and HOM (n=17) mice were submitted, after an initial stabilization period of 50 minutes, to 40 minutes of global ischemia followed by 60 minutes of reperfusion. During ischemia, hearts were immersed in hypoxic Krebs solution at 37ºC (in mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25, KH_2PO_4 1.2, and sucrose 11, pH 7.4, bubbled with 95% N_2 : 5% O_2). In addition to functional data, myocardial cell death was determined by lactate dehydrogenase (LDH) release (n=8 for each genotype) during the entire reperfusion period, and by infarct size measurement by TTC staining at the end of the experiment, in all hearts (Figure 7).

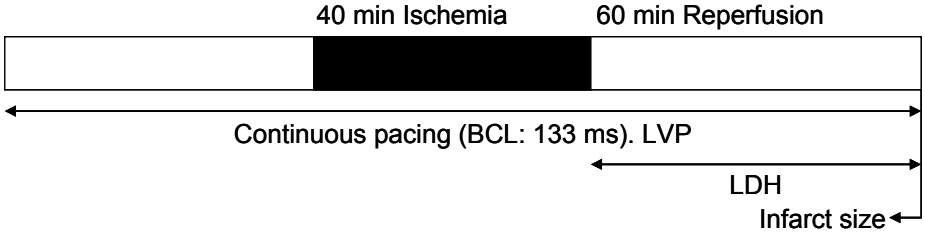
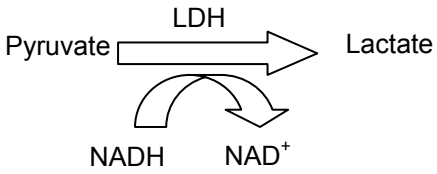


Figure 7.- Experimental protocol used to assess the effect of replacement of Cx43 by Cx32 (i.e., mimicking a reduction of maximal unitary conductance of connexin channels) on myocardial tolerance to ischemia-reperfusion injury in isolated mice hearts.

4.1.2.1- LDH release

Coronary effluent samples were taken at different time points during reperfusion, to spectrophotometrically analyze LDH release, as previously described (Rodriguez-Sinovas et al., 2004). LDH release is highly correlated with cell death. Sarcolemmal rupture causes release of the cytoplasmic content of dead cardiomyocytes, including this enzyme, that is quickly washed by the coronary effluent. LDH measurement is based on the following reaction:



The enzymatic assay was performed at 37ºC in 96-wells plates, by 1/5 dilution of coronary effluent samples in phosphate buffer (in mM: $K_2HPO_4 \cdot 3H_2O$ 61.4, KH_2PO_4 58.6, pH 7.4), in the presence of NADH 310 μM and sodium pyruvate 1 mM. The absorbance decay was measured in a spectrophotometer (Multiskan FC, Thermo Scientific, United States) at 320 nm, during 5 minutes. LDH catalytic concentration was calculated using the NADH molar absorptivity coefficient, and values were normalized respect to time (depending on flow) and dry ventricular weight, measured after 24 hours of dehydration at 100ºC.

4.1.2.2- Infarct size quantification. TTC staining

Infarct size was measured at the end of reperfusion by TTC staining. Hearts, lacking both atria and great vessels, were cut in cross sections perpendicular to the long cardiac axis, and incubated for 15 minutes at 37ºC in TTC, as previously described (Garcia-Dorado et al., 1997; Rodriguez-Sinovas et al., 2004). TTC is reduced by molecules present in living cells, such as NADH, leading to a red pigment precipitate.

This allows differentiation between dead (non-stained, and thus, seen in white) and alive (stained in red) tissue (Ferrera et al., 2009). Cardiac sections were weighted and photographied using a digital camera. Total sectional area and area of necrosis was measured in each ventricular slice using a commercially available software (Image-Pro Plus 4.5, Media Cybernetics-Olympus optical, Germany). Infarct size was expressed as percentage of total ventricular weight.

4.1.3- Effects of replacement of Cx43 by Cx32 on cardiac susceptibility to preconditioning protection

To study the effects of replacement of Cx43 by Cx32, a situation mimicking a reduction of maximal unitary conductance of connexins channels, on cardiac susceptibility to preconditioning protection, three different protocols were carried out in isolated hearts from WT, HET and HOM animals. Control hearts (n=9-11 /genotype) were submitted to 40 min of ischemia followed by reperfusion without any other intervention. Protection by IPC was tested in isolated hearts (n=9-11 /genotype) submitted to four cycles of 3.5 minutes of ischemia and 5 minutes of reperfusion, prior to the prolonged index ischemia. On the other hand, pharmacological preconditioning protection was analyzed by administration of Dzx (50 μM) 10 minutes before ischemia-reperfusion (n=9/ genotype). In addition to functional data, coronary effluent samples were obtained during the entire reperfusion period for analysis of LDH release, and infarct size was measured in all hearts at the end of the experiments (Figure 8).

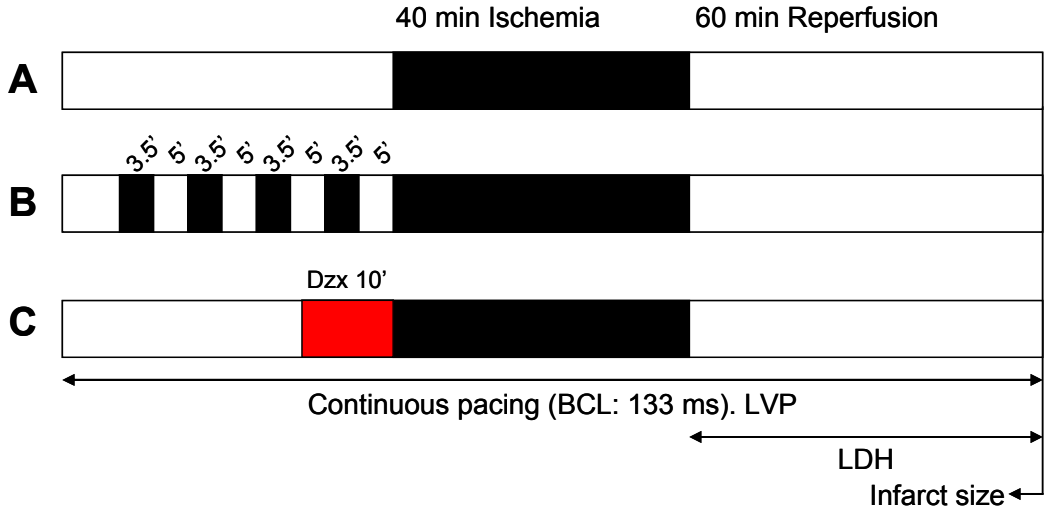


Figure 8.- Experimental protocols used to assess the effect of replacement of Cx43 by Cx32 (i.e., mimicking a reduction of maximal unitary conductance of connexin channels) on cardiac susceptibility to preconditioning protection. Hearts were submitted to 40 minutes of global ischemia followed by reperfusion under control conditions (A), or after pre-treatment with IPC (B) or pharmacological preconditioning with Dzx (C).

Additionally, a group of 9 HOM hearts were pre-treated with cariporide (7 μM) for 10 minutes just before ischemia-reperfusion. This group served as a positive control for cardioprotection in animals with this genotype. The mechanism of the protective effect of cariporide has been suggested to be independent of both GJ intercellular communication and Cx43 expression (Klein et al., 2000).

4.2- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

Treatment with 4-OHT in $Cx43^{Cre\text{-}ER(T)/fl}$ mice allows to assess the effects of a marked reduction in Cx43 expression on myocardial energetic metabolism, resistance to ischemia-reperfusion injury and protection by preconditioning. The use of this model is of importance, as it permits to determine whether the presence of Cx32 contributed to the findings obtained in our Cx43KI32 mice model. Whereas $Cx43^{Cre/fl}$ animals express half of the normal Cx43 content under baseline conditions, as compared with their corresponding controls $Cx43^{fl/fl}$, 4-OHT pre-treated $Cx43^{Cre/fl}$ animals contain only a residual 5-10% expression of the protein (Eckardt et al., 2004).

4.2.1- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on energetic metabolism

Metabolite concentration was first analyzed in cardiac extracts by ^{1}H-NMR spectroscopy (n=8 for each group). The protocols used to obtain lyophilized cardiac extracts and ^{1}H-NMR spectra were the same as described for Cx43KI32 mice in point 4.1.1.1 of material and methods.

PCr and ATP kinetics were also determined in isolated, Langendorff-perfused, hearts from $Cx43^{fl/fl}$ mice treated with vehicle and from $Cx43^{Cre/fl}$ mice treated with 4-OHT (n=4 for each group) by ^{31}P-NMR spectroscopy. Spectra were obtained and analyzed as previously described in point 4.1.1.2 of material and methods.

4.2.2- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on myocardial tolerance to ischemia-reperfusion injury

The role of Cx43 in myocardial tolerance to ischemia-reperfusion injury was analyzed in 35 hearts (n=8-9 /group) submitted to 40 min of global ischemia followed

by 60 min of reperfusion. In addition to functional data, LDH release was measured during the entire reperfusion period, and infarct size determined by TTC staining at the end of the experiment, as described in point 4.1.2 of material and methods (Figure 9).

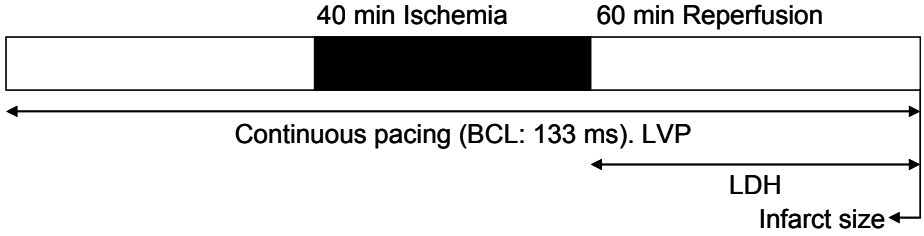


Figure 9.- Experimental protocol used to assess the effect of a marked reduction in Cx43 expression on myocardial tolerance to ischemia-reperfusion injury in isolated mice hearts.

4.2.3- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on cell death in isolated cardiomyocytes

In a recent study, we had demonstrated that the increased tolerance to myocardial ischemia-reperfusion injury observed in Cx43KI32 mice was, at least in part, independent of GJ intercellular communication (Rodriguez-Sinovas et al., 2010). This was based in the fact that isolated cardiomyocytes, that no longer form functional GJ after isolation, were also partially protected against simulated ischemia and reperfusion. Therefore, we decided to analyze whether the presence of Cx32 had contributed to this effect. Cardiomyocytes from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, either treated with vehicle or 4-OHT (n=8-12 samples /group), were freshly isolated by collagenase perfusion, as previously described (Li et al., 2004). Isolated cardiomyocytes were plated on 12mm WillCo dishes (WillCo Wells, Holland) precoated with laminin, and incubated for 15-30 minutes at 37ºC in normoxic, glucose-containing, buffer (in mmol/L: NaCl 140, KCl 3.6, $MgSO_4$ 1.2, $CaCl_2$ 1, Hepes 20 and glucose 5, pH 7.4, oxygenated with 100% O_2). Cells were, then, submitted to 15 min of simulated ischemia by incubation in an hypoxic chamber ($Invivo_2$ Workstation (atmosphere containing 0% O_2 and 5% H_2, at 37ºC), Ruskin, United Kingdom) in a glucose-free buffer (in mmol/L: NaCl 140, KCl 3.6, $MgSO_4$ 1.2, $CaCl_2$ 1 and Hepes 20, pH 6.4), supplemented with resazurin (4 μM), ascorbic acid (100 μM), dithionite (0.5 mM) and superoxide dismutase from bovine erythrocytes (100 U/ml). Reperfusion (37ºC) was simulated by removal of culture dishes from the hypoxic chamber and replacement of the glucose-

free buffer by the glucose-containing medium. After 15 minutes of simulated reperfusion, WillCo dishes were incubated in normoxic buffer containing 0.04% Trypan blue, for two minutes, and analyzed by optical microscopy (BX41 microscope, Olympus, Taiwan). Images were captured using a digital camera (Olympus ColorView IIIu) connected to the microscope. Dead cardiomyocytes were stained in blue, due to free entrance of the dye through broken sarcolemma. Cell death was expressed as percentage of total cell number counted in each WillCo dish. Control, normoxic, cells were continuously incubated in glucose-containing buffer at 37ºC, changed every 15 minutes (Figure 10).

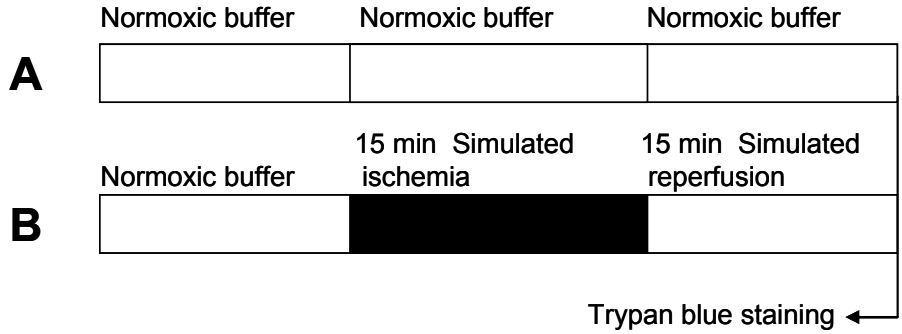


Figure 10.- Experimental protocols used to assess the effect of a marked reduction in Cx43 expression on cell death in isolated cardiomyocytes from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, either treated with vehicle or 4-OHT, and submitted to normoxic conditions (A) or to simulated ischemia-reperfusion (B).

4.2.4- Activation of cytosolic protective pathways during ischemia-reperfusion injury

Results obtained in our previous sections pointed to an increased resistance to ischemia-reperfusion injury in Cx43-deficient animals. However, our data do not allow to discard whether these findings are in fact independent of Cx43-deficiency, and related to an endogenous, baseline, activation of protective cytosolic signaling pathways in our transgenic models. Both RISK and SAFE signaling cascades have been suggested to be involved in protective pathways in several animal models, and activation of different kinases through phosphorylation has been associated with reduced infarct size (Schulz et al., 2001; Yellon and Downey, 2003; Lecour, 2009; Cohen and Downey, 2011). Thus, we aimed to analyze whether both signaling cascades were basally activated under normoxic conditions in $Cx43^{Cre/fl}$ mice treated with 4-OHT, as compared with the remaining groups. Activation of the RISK pathway was analyzed by

assessing phosphorylation levels of Akt, ERK-1/2, and GSK3β. On the other hand, STAT3-phosphorylation was determined to assess an involvement of the SAFE protective pathway.

Isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice treated with vehicle or 4-OHT were submitted to 44 minutes of normoxic perfusion (n=5-6 /group). Immediately after the perfusion period, hearts were quickly frozen in liquid N_2. Frozen hearts were, then, homogenized and total cardiac extracts were obtained using the same protocol described in point 2.2.2.1 of material and methods. Western blot analysis was performed to assess the activation state of Akt, ERK-1/2, GSK3β and STAT3, using a similar procedure to that described in point 2.2.2.3 of this part of the thesis, but with some modifications. First, membranes were incubated with antibodies raised against the phosphorylated forms of Akt, ERK-1/2, GSK3β and STAT3, and then, to total forms of the same proteins. To better visualize signals, and conserve phosphorylated antigens, blockade of unspecific binding was performed with a solution of TBS-T containing 5% Phosphoblocker (Cell Biolabs, United States). Membranes were incubated overnight in a cold chamber (4ºC) with one of the following rabbit antibodies raised against phosphorylated forms of the proteins: anti phospho-Akt, phospho-ERK-1/2, phospho-GSK3β or phospho-STAT3 (9271, 9101, 9323 and 9131, respectively, Cell Signaling, United States, dilution 1:500), followed by incubation with a secondary anti-rabbit antibody (Thermo Scientific-Pierce Protein Research Products, dilution 1:2000). Both primary and secondary antibodies were diluted in TBS-T containing 3% phosphoblocker. Once optical density was assessed, antibodies were removed from membranes by stripping and they were incubated again with additional rabbit antibodies in order to quantify total expression of the proteins: Akt, ERK-1/2, GSK3β or STAT3 (9272, 4695, 9315 and 9132, respectively, Cell Signaling, dilution 1:1000). Antibodies raised against total forms of the proteins were diluted in TBS-T containing 10% of Superblock Blocking Buffer. The secondary anti-rabbit antibody (Thermo Scientific-Pierce Protein Research Products, dilution 1:2000) was diluted in TBS-T added with 5% powdered skimmed milk. The ratio between phosphorylated and total form was determined, whose magnitude correlates with the activation state of these kinases.

4.2.5- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on cardiac susceptibility to preconditioning protection

The consequences of a marked reduction in the number of available connexin channels on protection by IPC was analyzed in isolated mice hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ animals, treated with vehicle or 4-OHT (n=8-9 /group), and subjected to either no intervention (control group), or four cycles of 3.5 minutes of ischemia and 5 minutes of reperfusion just before the prolonged index ischemia (IPC group).

As our results demonstrated that infarct size following ischemia-reperfusion was highly reduced in $Cx43^{Cre/fl}$ mice treated with 4-OHT, protection by pharmacological preconditioning with Dzx was only analyzed in mice treated with vehicle. Thus, 16 additional hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with vehicle (n=8 /group), were submitted to perfusion with Krebs buffer containing 50 μM of Dzx for 10 minutes just before ischemia. Protocols were similar to those described in point 4.1.3 of material and methods (Figure 11).

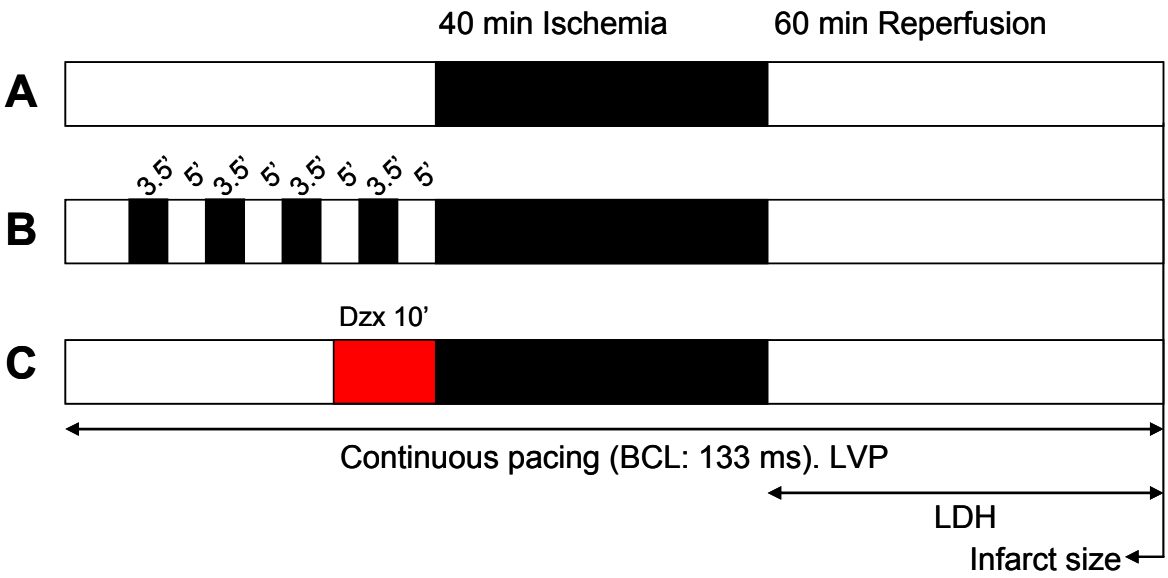


Figure 11.- Experimental protocols used to assess the effect of a marked reduction of available connexin channels on cardiac susceptibility to preconditioning protection. Hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, either treated with vehicle or 4-OHT, were submitted to control conditions (A) or IPC (B) before index ischemia. Pharmacological preconditioning with Dzx (C) was analyzed in $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice treated with vehicle.

4.2.6- Activation of cytosolic protective pathways during IPC in animals with reduced Cx43 expression

Lack of protection by preconditioning in Cx43-deficient animals might be due to absence of activation of cytosolic RISK and SAFE protective signaling pathways in this group. Thus, we aimed to analyze whether these signaling pathways were differentially activated by IPC in $Cx43^{Cre/fl}$ mice treated with 4-OHT. Isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, either treated with vehicle or 4-OHT, were submitted to four cycles of 3.5 minutes of ischemia and 5 minutes of reperfusion (n=5-6 /group). Immediately after the last preconditioning cycle, hearts were quickly frozen in liquid N_2 (Figure 12). Frozen hearts were, then, homogenized and total cardiac extracts were obtained using the same protocol described in point 2.2.2.1 of material and methods. Expression and phosphorylation state of kinases involved in RISK and SAFE protective pathways was analyzed by western blot and compared with that found in the normoxic hearts analyzed in point 4.2.4 of material and methods.

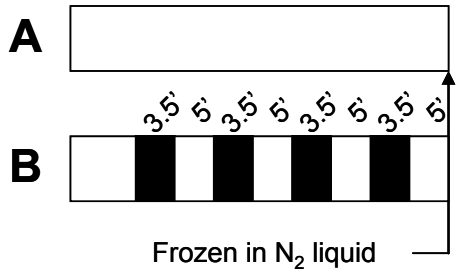


Figure 12.- Experimental protocols used to assess expression and phosphorylation of kinases involved in RISK and SAFE protective pathways. Hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, either treated with vehicle or 4-OHT, were submitted to normoxic perfusion (A) or IPC cycles (B).

4.3- Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on the incidence of ventricular arrhythmias, and on passive and active myocardial electrophysiological properties, during ischemia-reperfusion

4.3.1- Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on ventricular arrhythmogenesis

The incidence of both spontaneous and inducible ventricular arrhythmias during normoxia and ischemia-reperfusion was assessed in isolated mice hearts by epicardial electrogram recordings. Electrograms were monitored using stainless steel electrodes (6491 unipolar pediatric temporary pacing lead, Medtronic, France) placed in the left

ventricular base and right atria. A reference electrode was immersed in the hypoxic Krebs solution surrounding the hearts. Bipolar electrogram signals between left ventricle and right atria were amplified, digitized at 20 kHz, and stored in a computer using the PowerLab/8Sp data-acquisition system and the software Chart 5.0 (ADInstruments).

4.3.1.1- Incidence of spontaneous and inducible ventricular arrhythmias during normoxia

Spontaneous and inducible ventricular arrhythmias during normoxia were analyzed after 20 minutes of equilibration in 21 hearts from Cx43KI32 mice (10 WT and 11 HOM) and 43 hearts from $Cx43^{Cre\text{-}ER(T)/fl}$ mice, treated with vehicle or 4-OHT (n=9-12 /group). Spontaneous ventricular arrhythmias were analyzed during 10 minutes of constant pacing from the cardiac apex, using rectangular pulses of 2.5 ms duration, and an amplitude 2.5 fold the diastolic threshold, at two different BCLs: 133 and 100 ms (Figure 13A).

Ventricular tachyarrhythmias were, then, induced using a protocol of programmed electrical stimulation, consisting in one to five extrastimuli (S2-S6), introduced after a train of 16 stimuli (S1) at BCL of 100 ms. Extrastimuli were added at an interval 5 ms longer than the effective refractory period, which was calculated by successive 5 ms reductions of the time interval between the train of 16 S1 and the first extrastimuli (S2). Refractory period was defined as the longest S1-S2 interval which failed to produce a propagated response at the electrode located in the ventricular base. When this initial phase was completed, the induction protocol continued with burst pacing, which consisted of 18 stimuli (S1) at BCLs of 80, 60, 40, and 30 ms. The complete induction protocol was carried out three consecutive times (intertrain interval: 10 seconds) (Figure 13B).

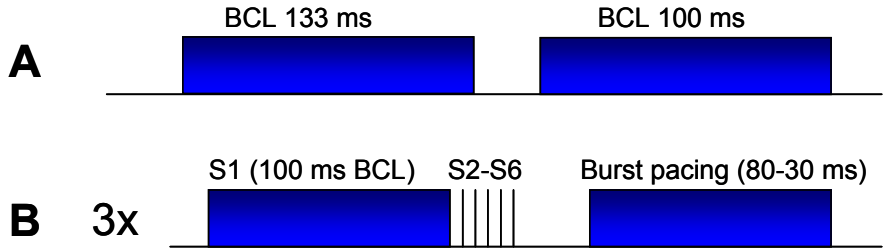


Figure 13.- Experimental protocols used to analyze the incidence of spontaneous ventricular arrhythmias (A) or induced ventricular tachyarrhythmias (B), after programmed electrical stimulation under normoxic conditions.

4.3.1.2- Incidence of spontaneous and inducible ventricular arrhythmias during regional ischemia-reperfusion

Spontaneous arrhythmias were monitored in hearts from Cx43KI32 mice (n=7 WT and 7 HOM) and $Cx43^{Cre\text{-}ER(T)/fl}$ mice, treated with vehicle or 4-OHT (n=5-7 /group), submitted to 20 minutes of regional ischemia and 60 minutes of reperfusion without pacing. Regional ischemia was performed by ligature of the left descending coronary artery using a 6/0 silk snare, placed 2-3 mm distal to the tip of left atria. Reperfusion was achieved by releasing the ligature. During ischemia, hearts were immersed in hypoxic Krebs solution at 37ºC. This short time of ischemia was selected to avoid major interferences of cell death in reperfusion arrhythmias. Successful coronary occlusion was verified by ST segment elevation in electrogram recordings, by a reduction in LVdevP, and by an increase in PP. All these changes reversed during reperfusion.

In addition to spontaneous arrhythmias, in these hearts, inducibility of ventricular tachyarrhythmias was tested 30 minutes after reperfusion, using the protocol of induction described in the previous point (Figure 14).

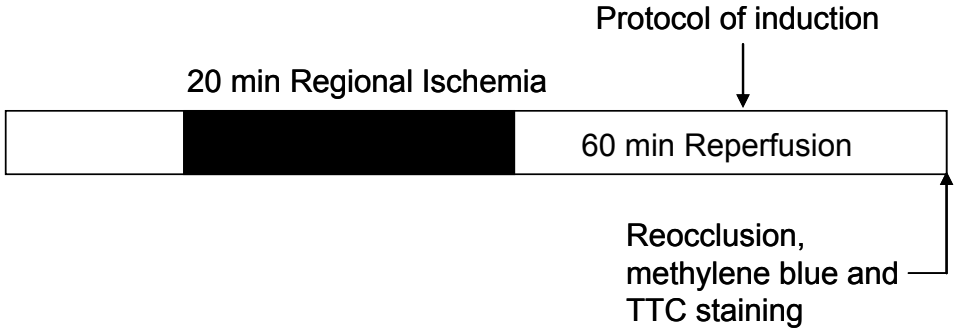


Figure 14.- Experimental protocol used to analyze the incidence of spontaneous ventricular arrhythmias during ischemia-reperfusion. Inducibility of ventricular tachyarrhythmias was tested 30 min after reperfusion, using the protocol described under normoxic conditions. After 60 minutes of reperfusion, left descending coronary artery was reoccluded, methylene blue was administered to measure area at risk, and infarct size was determined by TTC staining.

At the end of the reperfusion period, infarct size, expressed as percentage of area at risk, was determined. The left descending coronary artery was reoccluded at the same location, and the size of the area at risk was measured after injection of 0.5 ml of a solution of 0.02% methylene blue through the aortic cannula. Hearts were quickly removed, cooled at 4ºC, and cut into five to six cross sections. Cardiac slices were weighted and imaged, together with a reference scale, under white light using a digital

camera. The area not stained by methylene blue was considered to be the area at risk. The left ventricular mass and mass of the area at risk was quantified from the digital images (Image-Pro Plus 4.5, Media Cybernetics-Olympus optical, Germany). The total mass of the left ventricle was measured by adding the weights of each slice, whereas the mass of the area at risk was calculated as $\sum$(area at risk$_i$ /area left ventricle$_i$ * weight$_i$), being i each individual slice. Area at risk was then expressed as percentage of total ventricular mass. Infarct size was determined in cardiac slices by TTC staining, as described in point 4.1.2.2 of material and methods.

Finally, additional hearts from Cx43KI32 mice (n=8 WT and 8 HOM) and Cx43$^{Cre\text{-}ER(T)/fl}$ mice, treated with vehicle or 4-OHT (n=5 from each group), were submitted to 60 minutes of regional ischemia without pacing. Inducibility of ventricular tachyarrhythmias was analyzed in these isolated hearts at minutes 5, 15, 30, 45 and 60 of ischemia, using the protocol of induction described in normoxic hearts. At the end of ischemia, the size of the area at risk was measured as described above. Infarct size could not be assessed due to the absence of reperfusion (Figure 15).

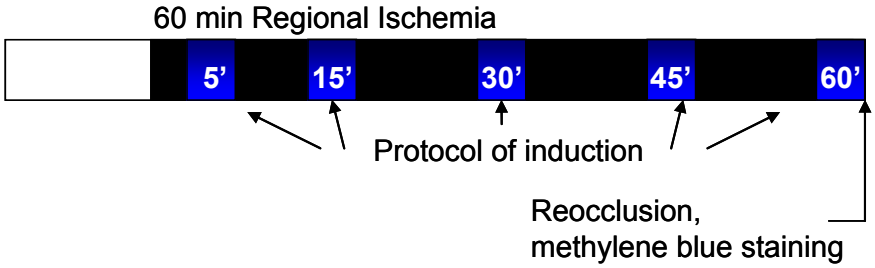


Figure 15.- Experimental protocol used to analyze inducibility of ventricular tachyarrhythmias during regional ischemia in isolated mice hears. Inducibility was tested using the protocol described during normoxia, at minutes 5, 15, 30, 45 and 60 after coronary occlusion.

4.3.2- Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on passive and active myocardial electrical properties

4.3.2.1- Conduction velocity

Ventricular conduction velocity was studied during ischemia in 23 isolated hearts from Cx43KI32 mice (n=14 WT and 9 HOM) and 24 cardiac preparations from Cx43$^{Cre\text{-}ER(T)/fl}$ mice, treated with vehicle or 4-OHT (n=6 from each group). After a period of stabilization, isolated hearts were submitted to 40 minutes of global ischemia and 60 minutes of reperfusion, while pacing from the cardiac apex at BCL of 133 ms (450 bmp). Hearts were immersed in hypoxic Krebs solution at 37ºC during the ischemic period.

Ventricular activation time was measured in unipolar electrograms, recorded by stainless steel electrodes (6491 unipolar pediatric temporary pacing lead, Medtronic, France) placed in the left ventricular base, as the time between the stimulus artifact and the onset of the QRS in the measuring ventricular electrogram. Signals were amplified, digitized at 20 kHz, and stored for later analysis using a PowerLab/8SP data-acquisition system and the software Chart 5.0 (ADInstruments). From activation times, and considering the distance between the stimulus site (cardiac apex) and the recording site (left ventricular base), we estimated ventricular conduction velocity. Using this method, the estimated conduction velocity represents an averaged velocity between both sites, as the pathway of activation is not known.

4.3.2.2- Passive myocardial electrical properties: tissue resistivity

Myocardial electrical impedance, including tissue resistivity and phase angle, was studied in the same previous 23 isolated hearts from Cx43KI32 mice (n=14 WT and 9 HOM) and 24 cardiac preparations from $Cx43^{Cre\text{-}ER(T)/fl}$ mice, treated with vehicle or 4-OHT (n=6 from each group). After a period of stabilization, isolated hearts were submitted to 40 minutes of global ischemia and 60 minutes of reperfusion, while pacing from the cardiac apex at BCL of 133 ms (450 bmp). Hearts were immersed in hypoxic Krebs solution at 37ºC during the ischemic period.

Measurement of myocardial electrical impedance is an overall estimation of the passive electrical properties of the myocardium, which includes the intra- and extracellular resistance and the membrane capacitance. Myocardial electrical impedance is better characterized by measuring its two components: the in-phase component of voltage (V) with respect to current intensity (I) (i.e., tissue resistivity (R), calculated by direct application of the Ohm's law, R=V/I), and the phase angle (θ). Because biological tissues are not purely resistive, the capacitance of cell membranes must be considered. Phase angle is determined from the time delay between the voltage and intensity curves, by applying the formula $\theta = 360 \cdot \Delta t \cdot f$, where Δt is the time delay and f the frequency of the injected current (Rodriguez-Sinovas et al., 2006b). Its absolute value depends on membrane capacitance and is influenced by extracellular, intracellular, and membrane resistances. Changes in myocardial electrical impedance were analyzed during the whole experiment using a probe consisting of a linear array of four platinum electrodes (interelectrode distance = 1 mm), placed in the left ventricular free wall, as previously described (Rodriguez-Sinovas et al., 2004) (Figure 16). An

alternating current of 10 μA was applied through the outer pair of electrodes at a frequency of 7 Hz. The in-phase components of voltage and phase angle were continuously recorded by the inner pair of electrodes and amplified in a high-input impedance lock-in amplifier (5110, Princeton Applied Research, United States). Ischemia is known to induce an initial slight change in both components of tissue impedance, followed by a second sharp increase in resistivity, and a decrease in phase angle. The onset of these sharp changes has been related to the onset of cell-to-cell electrical uncoupling (Kleber et al., 1987; Padilla et al., 2003).

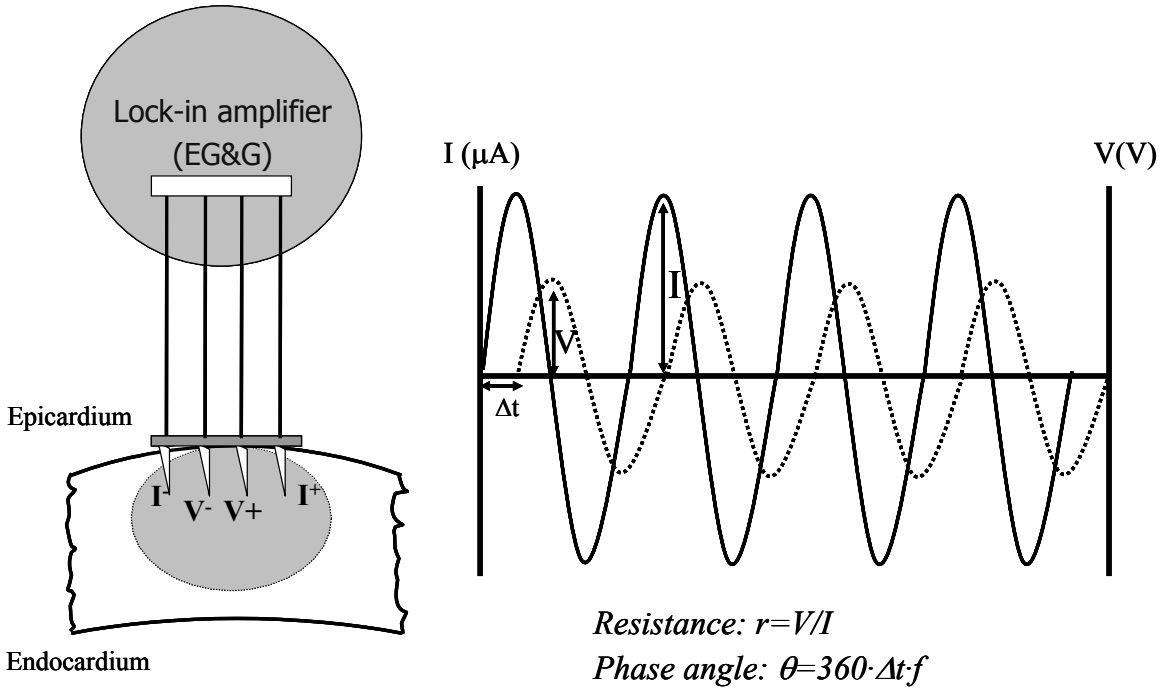


Figure 16.- Myocardial electrical impedance recordings in isolated mice hearts. An alternating current (I) was injected in the tissue by the two external electrodes of the linear array, whereas voltage difference (V) was measured by the internal ones. The time gap between both curves is shown on right. Δt: change in time; f: frequency (Modified from Rodriguez-Sinovas et al., 2006b).

4.3.2.3- Active myocardial electrical properties: transmembrane action potentials

Transmembrane action potentials were recorded from the left ventricular free wall in 6 Cx43KI32 mice (n=3 WT and 3HOM) and 6 $Cx43^{Cre\text{-}ER(T)/fl}$ mice (3 hearts from $Cx43^{fl/fl}$ mice treated with vehicle and 3 preparations from $Cx43^{Cre/fl}$ animals treated with 4-OHT). Isolated hearts were perfused in a modified Langendorff system, in which hearts were placed in horizontal position and pinned to a silicon membrane. Pacing was applied from the cardiac apex at BCL 133 ms (rectangular pulses of 2.5 ms duration and twice the diastolic threshold). Recordings were obtained using floating

glass microelectrodes filled with KCl (3 M) and a tip resistance between 25 and 35 MΩ, as previously described (Rodriguez-Sinovas et al., 2004). Microelectrodes were connected through Ag-AgCl interfaces to high-input impedance amplifiers, consisting in a dual microelectrode amplifier (VF102, Biologic, France), and a dual electrometer (IS100, Biologic as well). Signals were displayed in an oscilloscope (CS-8010, Kenwood, United States), digitalized at 40 Hz, and stored in a computer using the PowerLab/8SP data-acquisition system and the software Chart 5.0 (ADInstruments). To reduce motion artifacts, and thus, improve impalements, blebbistatin (10 μM) was added to the perfusion solution. Blebbistatin has been described to uncouple cardiac excitation and contraction through inhibition of myosin II isoforms, without inducing changes in action potential characteristics (Fedorov et al., 2007). Resting membrane potential, action potential amplitude, action potential duration at 25%, 50% and 75% of repolarization, and the maximal rate of rise of action potential upstroke (dV/dt_{max}) were measured for each stable impalement (5 impalements /heart).

5. Statistics

Data are expressed as mean ± standard error (SE). Differences were analyzed by Student's *t* test, when two groups were compared, or one-way analysis of variance (ANOVA) and Tukey's *post hoc* tests, when more than two groups were compared. Differences in temporal evolution were assessed by repeated-measures ANOVA (MANOVA) and Dunnett's *post hoc* tests. The effects of two different manoeuvres and their interaction were analyzed by two-way ANOVA.

For those variables that did not follow a Gaussian distribution, as is the case of the number of both spontaneous and induced ventricular arrhythmias, differences were assessed by non-parametric Mann-Whitney U or Kruskal-Wallis tests, as needed, whereas incidences were evaluated by Fisher's exact test or Chi-square test (χ^2).

Statistical analyses were performed with the software SPSS 15.0 (SPSS Inc., United States). Differences were considered to be significant when $p<0.05$.

Results

1. Effects of replacement of Cx43 by Cx32 in Cx43KI32 mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

1.1- Cx43KI32 mice model

Replacement of Cx43 by Cx32 induced some changes in the physical appearance of these animals, as previously described (Plum et al., 2000). Thus, HOM mice had a reduction in body and cardiac weight, as compared with WT and HET mice (Table 1). However, the ratio cardiac weight /body weight was similar in all groups, as also happened with LVEDP, LVdevP and PP.

Table 1. Body and cardiac weight, and baseline cardiac function parameters of isolated mouse hearts from WT, HET and HOM Cx43KI32 animals.

	WT mice (n=22)	HET mice (n=16)	HOM mice (n=17)
Body weight (g)	32.07 ± 1.14	31.59 ± 2.01	21.79 ± 0.83*
Cardiac weight (mg)	121.83 ± 4.64	116.82 ± 5.29	88.72 ± 4.08*
Ratio CW/BW (mg/g)	3.84 ± 0.12	3.79 ± 0.14	4.07 ± 0.10
LVEDP (mmHg)	6.22 ± 1.65	7.81 ± 1.65	7.15 ± 1.18
LVdevP (mmHg)	71.33 ± 4.89	85.25 ± 9.95	79.87 ± 4.93
PP (mmHg)	93.28 ± 8.29	83.25 ± 9.98	92.01 ± 10.41

PP and LVP were measured in 8 experiments /genotype. CW: cardiac weight; BW: body weight. * ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. results obtained in WT and HET mice.

PCR analysis of DNA extracted from tail samples allowed us to determine the genotype of each individual mouse. Cx43 allele gave an amplicon of 650 bp, whereas Cx32 allele was detected as an amplicon of 564 bp (Figure 17A).

Confocal images of mouse hearts showed a similar expression pattern for Cx43 and Cx32 in cardiac slices from WT and HOM mice, respectively, whereas both isoforms were equally observed in samples from HET mice. Both connexin isoforms were mainly located at the cell poles, within the intercalated disks (Figure 17B). No signal was detected when the primary antibody was omitted (negative control samples, images not shown).

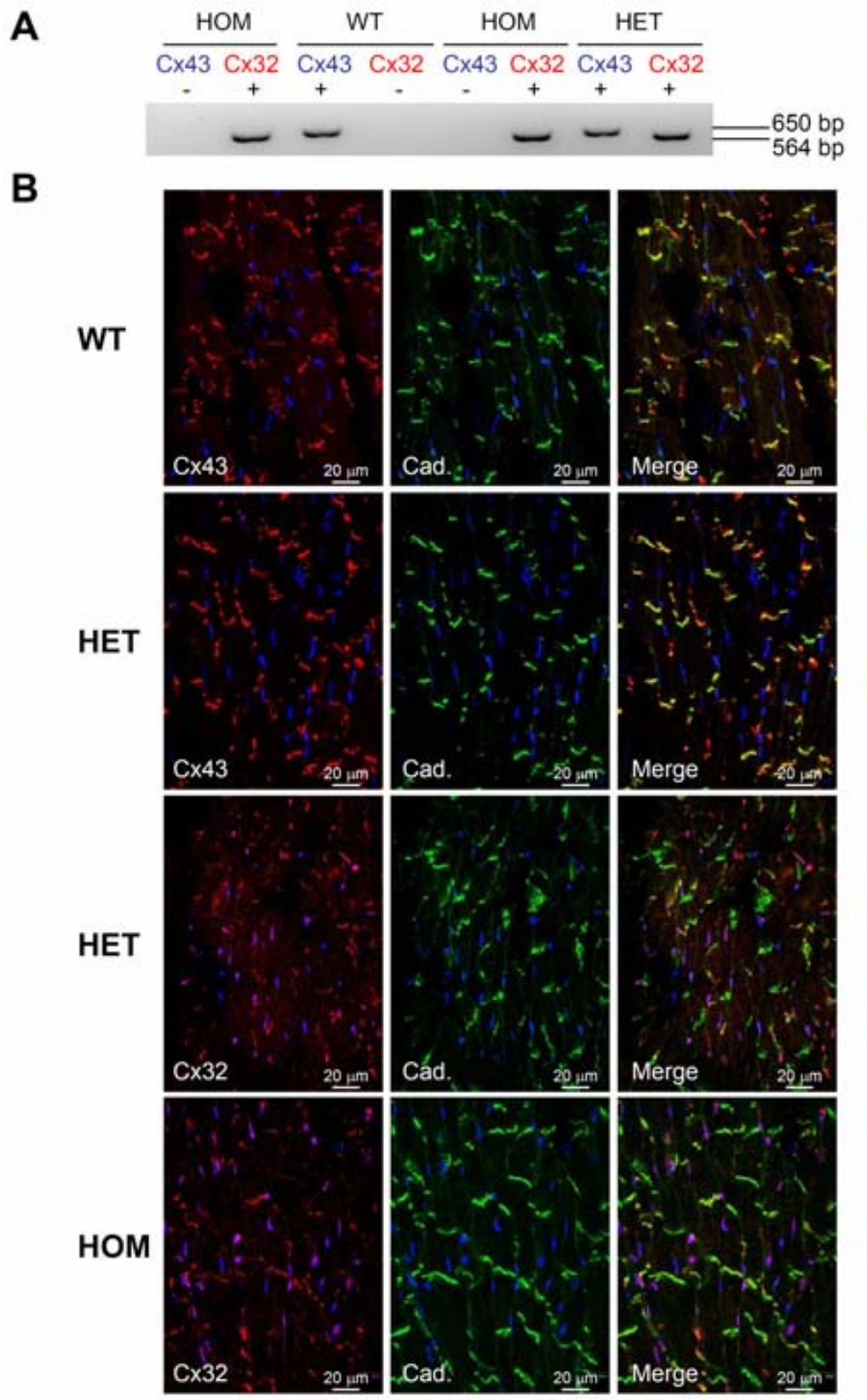


Figure 17.- (A) PCR analysis of DNA from Cx43KI32 mice. Cx43 allele gave an amplicon of 650 bp, whereas Cx32 allele was detected as an amplicon of 564 bp. (B) Confocal images showing in red Cx43 and Cx32 expression in cardiac sections from WT, HET and HOM animals. Intercalated disks were marked with an antibody against pan-cadherin (Cad., green) and nuclei with Hoeschst 33342 (blue).

1.2- Effects of replacement of Cx43 by Cx32 on energetic metabolism

Replacement of Cx43 by Cx32 induced marked changes in the intracellular concentration of some metabolites, as determined by ^{1}H-NMR spectroscopy of cardiac tissue extracts (Table 2). Lactate content was significantly increased in hearts from HOM mice, as compared with WT, suggesting increased glycolysis. Cardiac tissue extracts from animals of this genotype also depicted a significant reduction in ATP concentration, indicating impaired energetic metabolism. This was further confirmed by ^{31}P-NMR spectroscopy of isolated, Langendorff perfused, hearts, showing a reduced PCr/ATP ratio in those from HOM mice (Figure 18).

Table 2. Concentration of selected intracellular metabolites, analyzed in cardiac tissue extracts from WT and HOM mice (in μmol /10 μmol creatine).

	WT (n=5)	HOM (n=6)
Total creatine (μmol /mg protein)	0.94 ± 0.08	0.81 ± 0.07
Lactate	3.23 ± 0.51	6.47 ± 1.59 *
Alanine	1.19 ± 0.16	1.37 ± 0.28
Succinate	0.97 ± 0.35	1.15 ± 0.48
Glucose	0.10 ± 0.06	0.38 ± 0.12
Taurine	27.17 ± 1.06	31.60 ± 2.93
Formate	1.12 ± 0.26	1.00 ± 0.27
ATP	1.28 ± 0.21	0.67 ± 0.19 *
ADP	0.74 ± 0.11	2.22 ± 0.37 *

* ($p<0.05$, Student's *t* test) indicates significant differences between values obtained in WT and HOM mice.

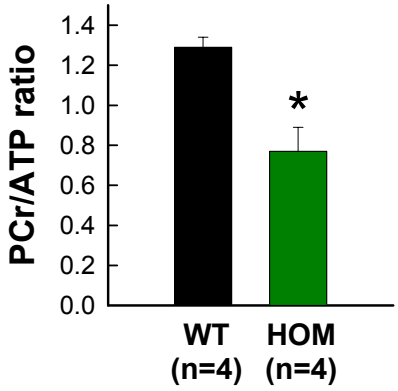


Figure 18.- PCr/ATP ratio measured by ^{31}P-NMR spectroscopy in isolated hearts from WT and HOM mice. * ($p<0.05$, Student's *t* test) indicates significant differences between both genotypes.

1.3- Effects of replacement of Cx43 by Cx32 on myocardial tolerance to ischemia-reperfusion injury

1.3.1- Left ventricular function

Global ischemia induced, in isolated mice hearts, a marked decrease in LVdevP, which reached a minimum two or three minutes later. Ischemic rigor contracture was detected as an abrupt increase in LVEDP, and occurred between minutes three and four after the onset of ischemia (Figure 19). Reperfusion, which gave rise to a quick recovery in PP, induced also a marked increase in LVEDP, reflecting hypercontracture (Figure 19 and 20A). Maximal hypercontracture was significantly attenuated in hearts from HOM, but not from HET mice, as compared with those from WT animals (Figure 20B). LVdevP increased during reperfusion to about 10% of the baseline value, without significant differences between groups, although recovery tended to occur earlier in hearts from HOM animals (Figure 20C).

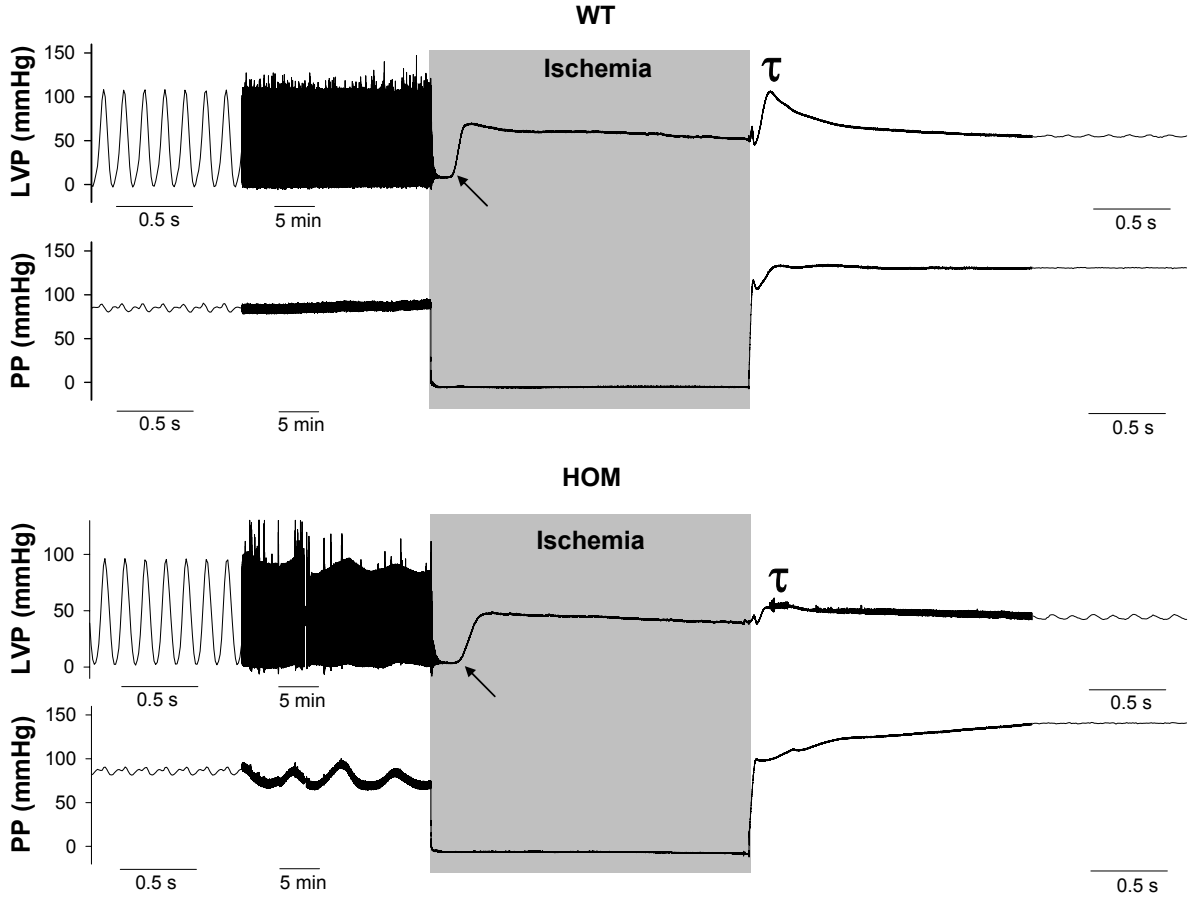


Figure 19.- Representative recording showing changes induced by ischemia-reperfusion in LVP and PP in isolated hearts from WT (above) and HOM (below) mice. Ischemic rigor contracture was observed as an increase in LVEDP at the beginning of ischemia (arrows), whereas hypercontracture was detected as the abrupt increase in LVEDP at the onset of reperfusion (τ), when PP was restored.

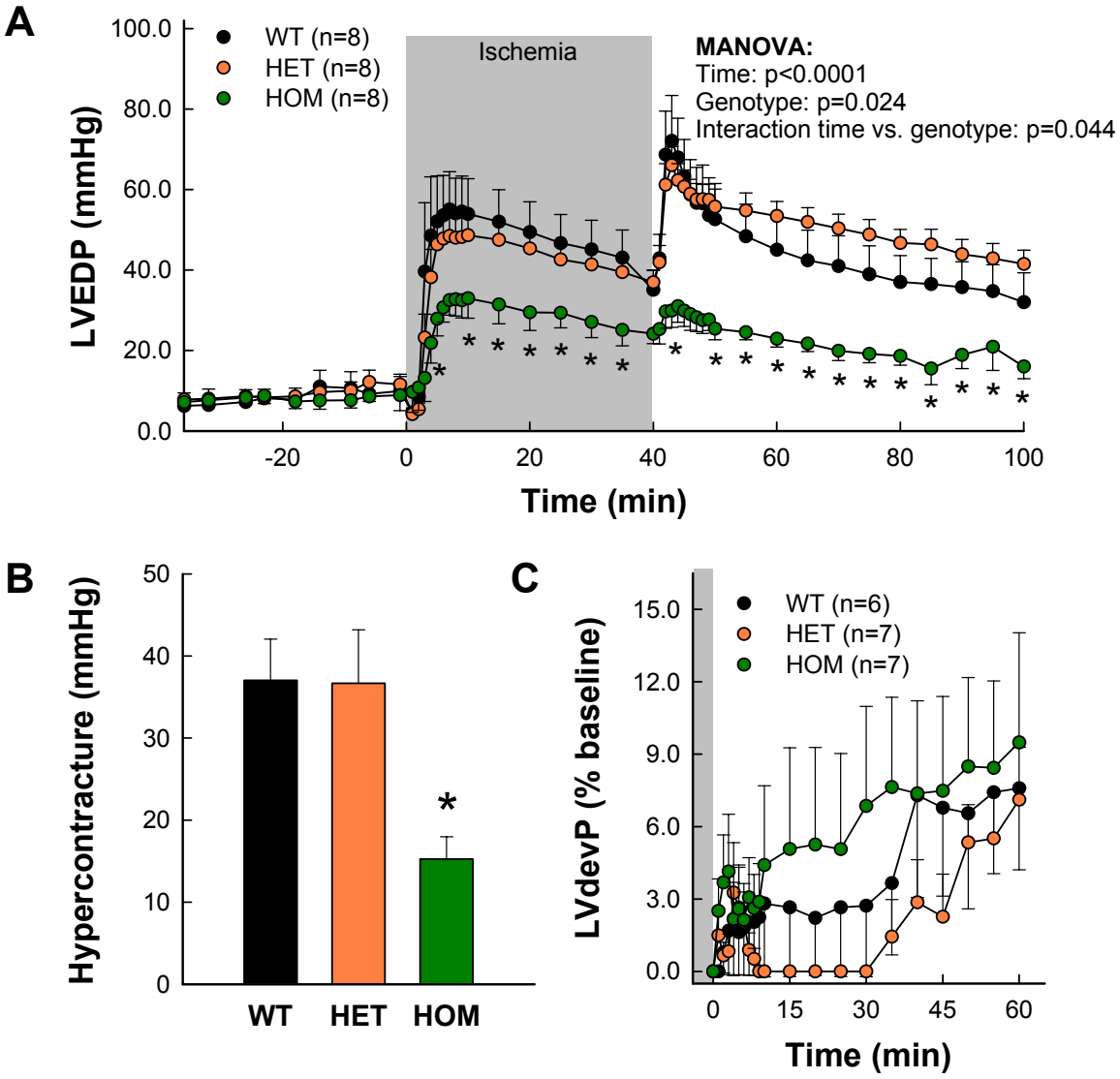


Figure 20.- (A) Changes in LVEDP observed in hearts from WT, HET and HOM mice submitted to ischemia-reperfusion. * ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences vs. all remaining groups. (B) Maximal hypercontracture in hearts from all genotypes, calculated as the difference between peak LVEDP at the beginning of reperfusion and its last value during ischemia. (C) Functional recovery during reperfusion, expressed as percentage of baseline LVdevP in isolated hearts from WT, HET and HOM mice. * ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. results obtained in hearts from WT animals.

1.3.2- Cell death

Cell death induced in all groups a marked release of LDH at reperfusion, peaking during the first minutes. However, LDH release at reperfusion and its cumulative value during the first 30 minutes of flow restoration were significantly reduced in hearts from HOM animals submitted to ischemia-reperfusion, as compared with those from WT and HET mice. These results, that correlated with a reduction in

infarct size in hearts from HOM animals, indicate a lower cell death in hearts from this genotype and, thus, an increased tolerance to ischemia-reperfusion injury (Figure 21). No differences in LDH release and infarct size were observed between sexes in any genotype.

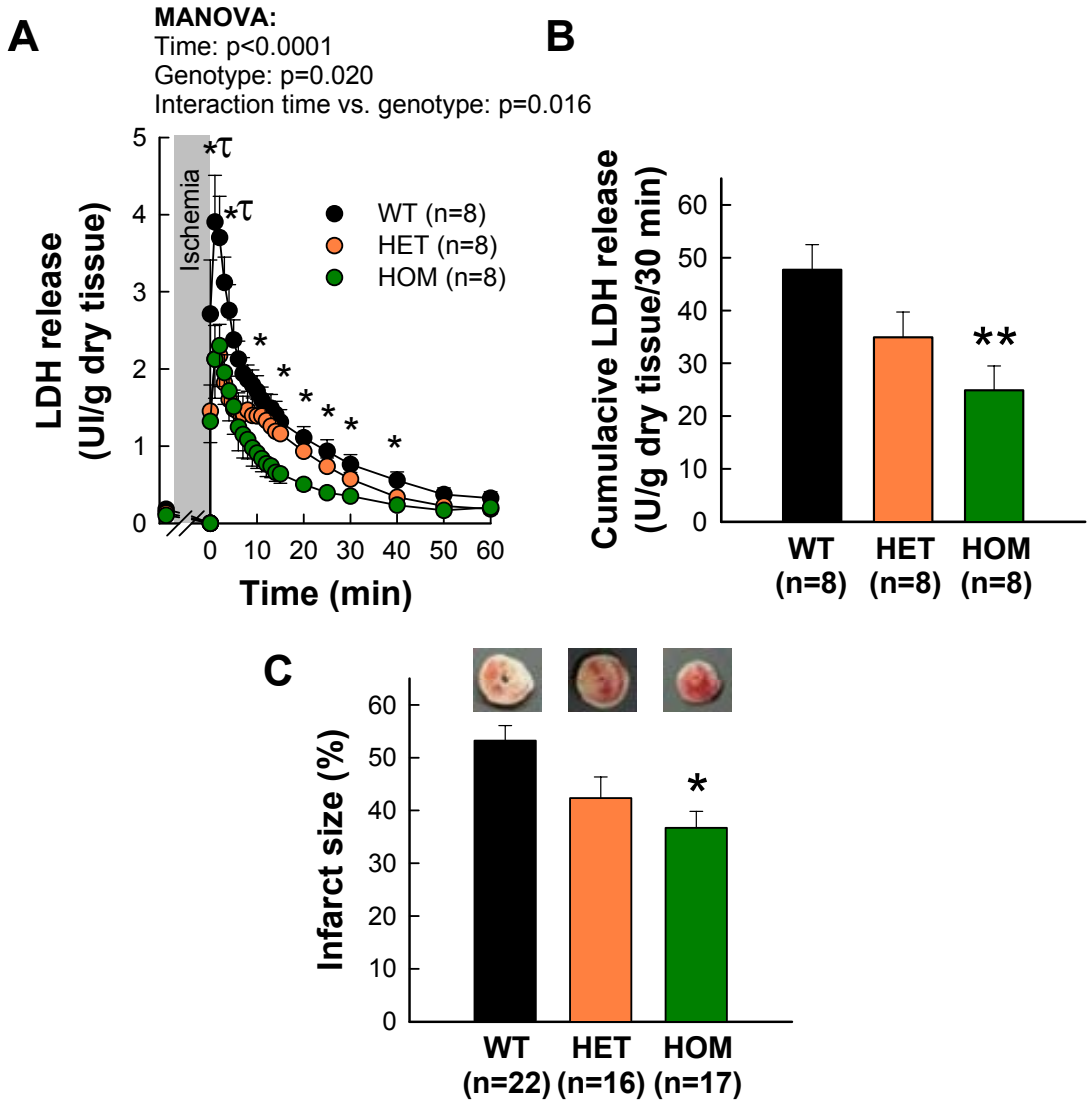


Figure 21.- LDH release during the complete reperfusion period (A) and its cumulative value during the first 30 minutes of flow restoration (B), in hearts from WT, HET and HOM mice submitted to 40 min of ischemia and 60 min of reperfusion. * and τ ($p<0.05$) indicate significant differences vs. all remaining groups, and between HET and WT animals, respectively, both assessed by MANOVA and Dunnett's *post hoc* tests. (C) Infarct size and representative images of cardiac slices stained with TTC, showing alive cells in red. Infarct size was measured in all experimental hearts from WT, HET and HOM mice submitted to the protocol of ischemia-reperfusion. * ($p<0.05$) and ** ($p<0.01$) indicate significant differences between HOM and WT mice, as assessed by ANOVA and Tukey's *post hoc* tests.

1.4- Effects of replacement of Cx43 by Cx32 on cardiac susceptibility to preconditioning protection

Both IPC and pharmacological preconditioning with Dzx afforded protection against ischemia-reperfusion injury in hearts from WT animals, as both manoeuvres reduced hypercontracture (Figure 22A), LDH release (Figure 23A) and infarct size (Figure 23D) in this genotype, compared with the control group. In addition, functional recovery at the end of reperfusion was significantly increased by IPC in these hearts (Figure 22B). In contrast, hearts from HET mice were protected by IPC, but not by Dzx (Figures 22 and 23), and both manoeuvres failed to induce protection in hearts from HOM animals (Figures 22 and 23). Additional experiments in hearts from this genotype showed that they still were able to be protected by cariporide pre-treatment at 7 μM (infarct size: 26.35 ± 2.79 % vs. 40.13 ± 2.69 % in control hearts, p=0.002, Student's *t* test).

A two-way ANOVA was performed for infarct size, LDH release, hypercontracture and functional recovery at the end of reperfusion. This statistical analysis showed a significant effect of genotype on infarct size, LDH release and hypercontracture, thus indicating reduced ischemia-reperfusion injury in hearts from HOM animals, together with a significant interaction between genotype and treatment (control, IPC or preconditioning with Dzx), suggesting that the degree of protection depends on genotype.

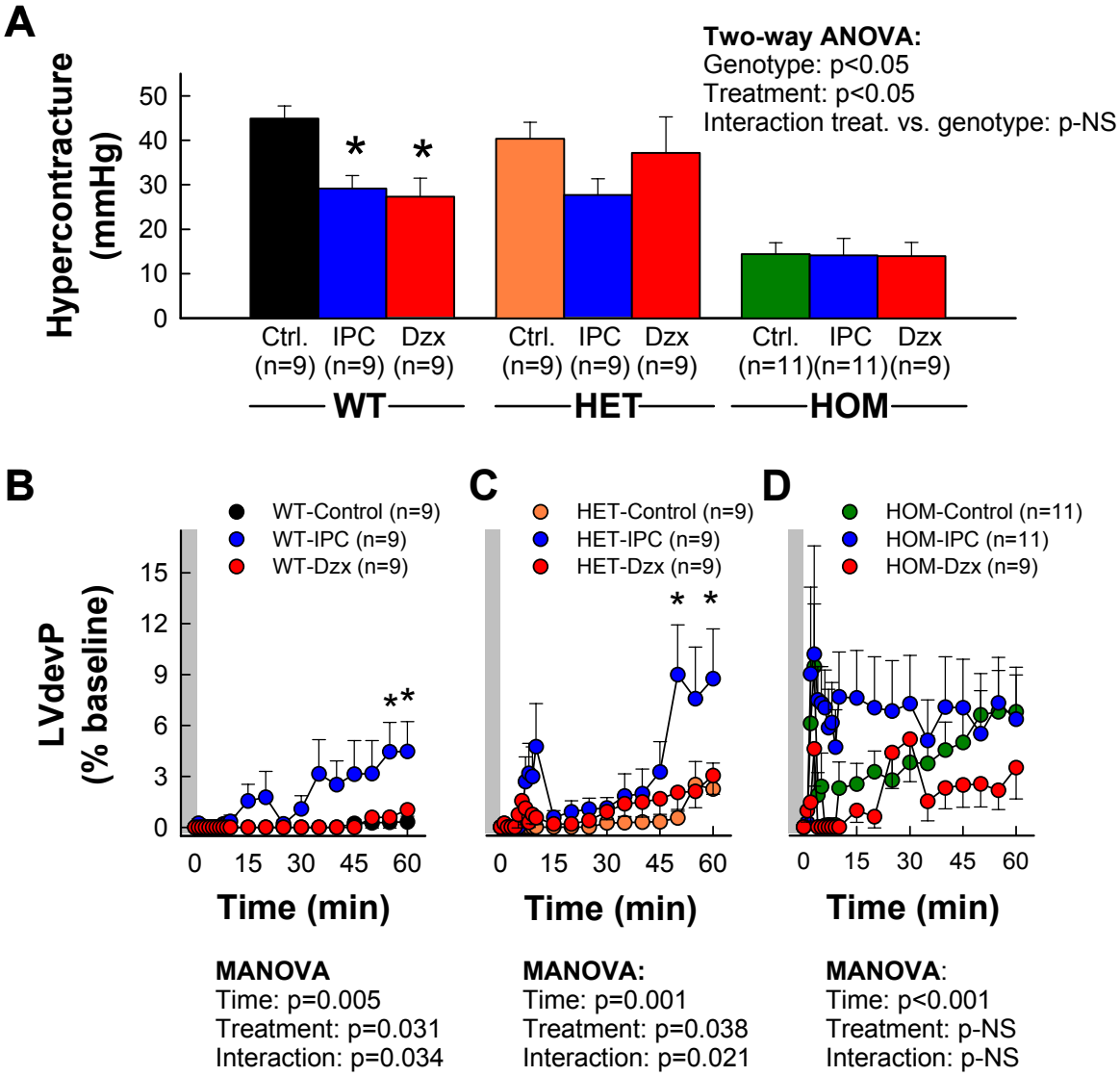


Figure 22.- (A) Maximal hypercontracture, calculated as the difference between LVEDP peak during reperfusion and its value at the end of ischemia, in isolated hearts from WT, HET and HOM mice submitted to ischemia-reperfusion under control conditions (Ctrl.), or after IPC or Dzx pre-treatment. * ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. the corresponding control group. (B-D) Functional recovery during reperfusion, calculated as percentage of baseline LVdevP, in isolated hearts from WT (B), HET (C) and HOM (D) animals submitted to ischemia-reperfusion, in the different treatment groups. * ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences vs. the corresponding control group.

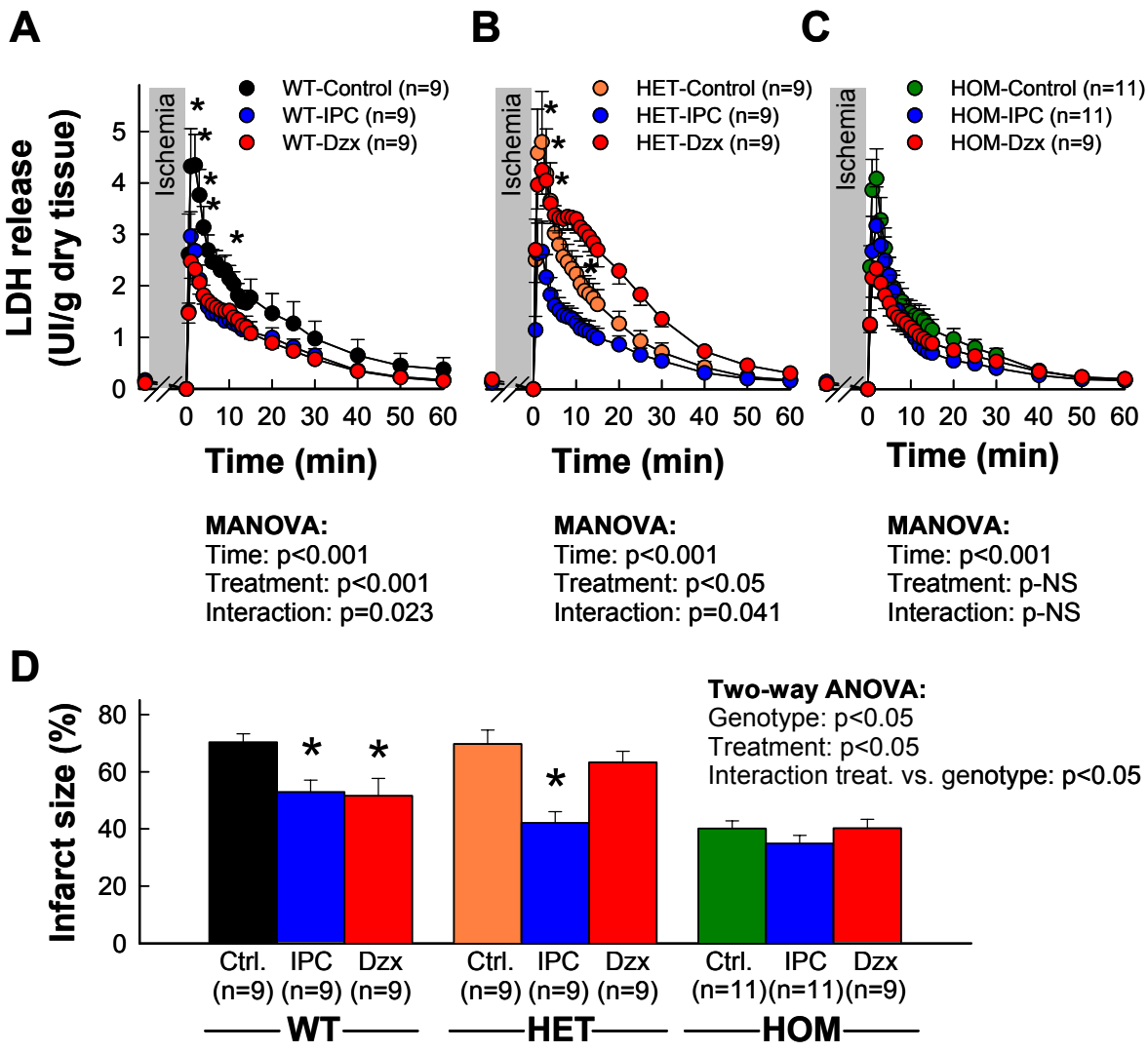


Figure 23.- LDH release during reperfusion in isolated hearts from WT (A), HET (B) and HOM (C) mice submitted to ischemia-reperfusion under control conditions, or after IPC or Dzx pre-treatment. * ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences between control hearts and those submitted to IPC and preconditioning with Dzx in WT mice, but only vs. the IPC group in HET animals. (D) Infarct size, analyzed by TTC staining, in isolated hearts from the three genotypes submitted to the three different treatments. * ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. the corresponding control group.

2. Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

2.1- $Cx43^{Cre\text{-}ER(T)/fl}$ mice model

A marked reduction in Cx43 expression in $Cx43^{Cre/fl}$ mice treated with 4-OHT did not induce significant differences regarding cardiac or body weight, or the ratio cardiac weight /body weight, as compared with $Cx43^{fl/fl}$ mice. Similarly, no differences were observed in LVEDP or PP. However, LVdevP was significantly increased in isolated hearts from $Cx43^{Cre/fl}$ mice treated 4-OHT, as compared with all remaining groups (Table 3).

Table 3. Body and cardiac weight, and baseline cardiac function parameters of isolated mouse hearts from $Cx43^{Cre\text{-}ER(T)/fl}$ animals.

	$Cx43^{fl/fl}$ + oil (n=26)	$Cx43^{fl/fl}$ + 4-OHT (n=18)	$Cx43^{Cre/fl}$ + oil (n=26)	$Cx43^{Cre/fl}$ + 4-OHT (n=16)
Body weight (g)	28.08 ± 0.96	27.70 ± 1.16	27.34 ± 1.00	24.23 ± 0.94
Cardiac weight (mg)	112.2 ± 4.63	103.16 ± 5.44	110.29 ± 5.52	101.80 ± 5.54
Ratio CW/BW (mg/g)	4.00 ± 0.09	3.71 ± 0.08	4.00 ± 0.09	4.18 ± 0.13
LVEDP (mmHg)	2.40 ± 1.31	4.12 ± 1.50	4.79 ± 1.42	1.52 ± 1.76
LVdevP (mmHg)	106.21 ± 4.86	100.51 ± 6.48	105.82 ± 4.25	136.55 ± 9.84**
PP (mmHg)	82.94 ± 3.74	86.46 ± 4.86	85.98 ± 3.02	84.62 ± 4.78

CW: cardiac weight; BW: body weight. ** ($p<0.01$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. results obtained in the remaining groups.

PCR analysis of DNA extracted from ear samples detected, in $Cx43^{Cre/fl}$ mice, an amplicon of 400 bp corresponding to the Cre-recombinase allele. Samples in which the amplicon did not appear were considered to be from control $Cx43^{fl/fl}$ mice (Figure 24A).

Western blot analysis of total fractions obtained from all hearts included in the different studies, demonstrated high expression levels of Cx43 in samples from $Cx43^{fl/fl}$ mice, either after treatment with vehicle (oil) or 4-OHT. In contrast, expression of Cx43 was reduced to about 56.26%, and to 4.28% of that calculated in $Cx43^{fl/fl}$ animals, in $Cx43^{Cre/fl}$ mice treated with oil and 4-OHT, respectively (Figure 24B and 24C). Cx43 depletion was associated with an increased mortality 14 days after the first induction

(2.17% mortality for $Cx43^{fl/fl}$ mice treated with oil, 2.63% mortality for $Cx43^{fl/fl}$ animals treated with 4-OHT, 0% mortality for $Cx43^{Cre/fl}$ mice treated with oil, and 38.46% mortality for $Cx43^{Cre/fl}$ animals treated with 4-OHT, $p<0.001$ vs. all previous groups, χ^2 test). Additional western blot analysis of mitochondrial fractions from cardiac samples showed similar reductions in Cx43 expression in $Cx43^{Cre/fl}$ mice treated with oil and 4-OHT (Figure 24D).

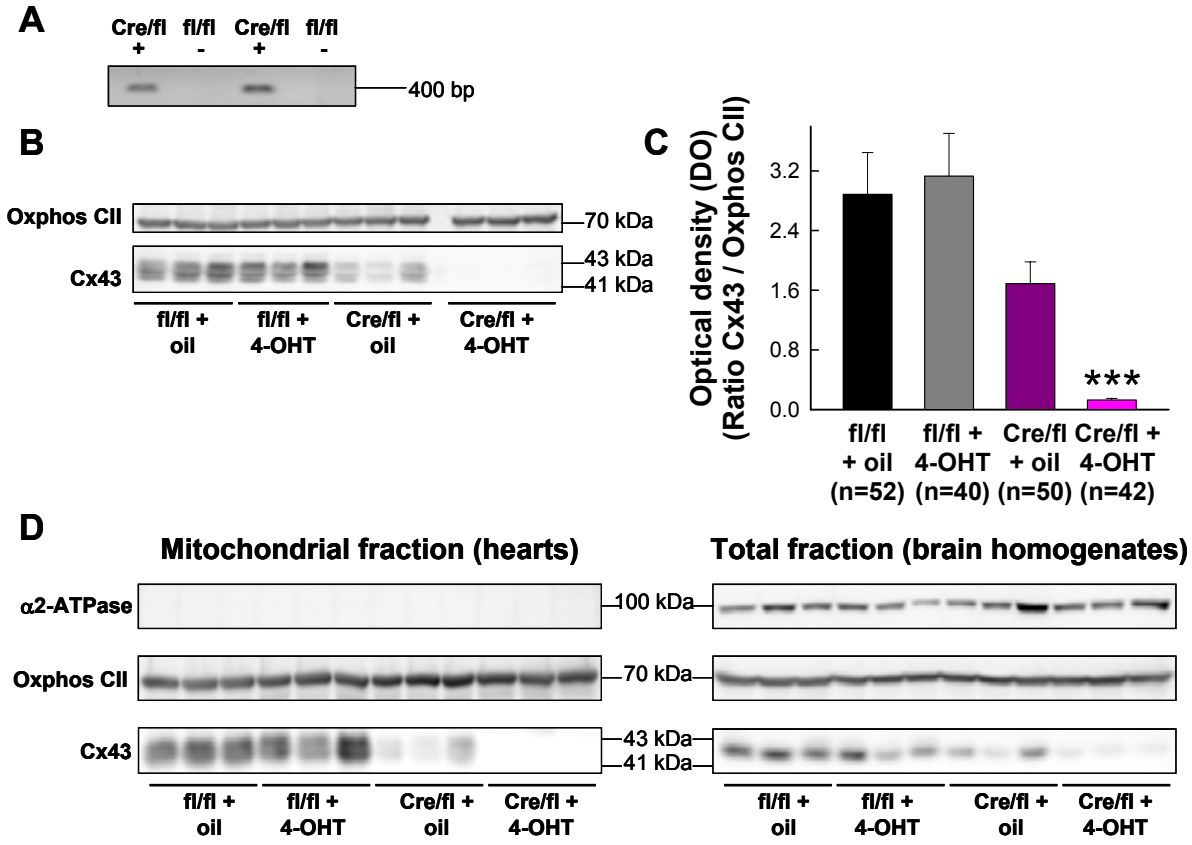


Figure 24.- (A) PCR analysis of ear samples from $Cx43^{Cre-ER(T)/fl}$ mice. Cre-recombinase allele gave an amplicon of 400 bp. These samples were considered to correspond to the $Cx43^{Cre/fl}$ genotype. Samples without the amplicon were considered to correspond to $Cx43^{fl/fl}$ mice. (B) Representative western blot, showing expression of Cx43 and Oxphos CII in hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice after treatment with oil or 4-OHT. (C) Expression of Cx43 calculated as the ratio between the optical density of Cx43 and Oxphos CII, measured in all experimental hearts. *** ($p<0.001$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. $Cx43^{fl/fl}$ mice. (D) Expression of Cx43, Oxphos CII and α2-Na^+/K^+ ATPase in mitochondrial fractions obtained 14 days after first induction, from cardiac samples from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with oil or 4-OHT. As a control, a western blot showing the expression of the same proteins in brain homogenates from the same animals is shown. Lack of α2- Na^+/K^+ ATPase in mitochondrial fractions indicates lack of contamination from plasma membrane. Abbreviations: fl/fl: $Cx43^{fl/fl}$; Cre/fl: $Cx43^{Cre/fl}$; α2-ATPase: α2 subunit of the sarcolemmal Na^+/K^+ ATPase.

Confocal images of mouse hearts from the four groups showed a normal expression and distribution of Cx43 in $Cx43^{fl/fl}$ mice treated either with vehicle or 4-OHT, being mainly located within the cell poles. In contrast, distribution of Cx43 in cardiac slides from $Cx43^{Cre/fl}$ mice treated with oil was heterogeneous, thus showing areas with normal Cx43 expression, but others in which only pan-cadherin, but not Cx43, was observed. Samples from $Cx43^{Cre/fl}$ mice treated with 4-OHT depicted a marked reduction in Cx43 expression, and also pan-cadherin seemed to be less expressed, and redistributed across the sarcolemmal membrane (Figure 25).

2.2- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre-ER(T)/fl}$ mice on energetic metabolism

A marked reduction in Cx43 expression was not associated with significant differences in the concentration of selected metabolites between the four experimental groups, as determined by ^{1}H-NMR spectroscopy of cardiac extracts, with the exception of taurine (Table 4). In contrast, ^{31}P-NMR spectroscopy of isolated, Langendorff perfused, hearts showed a reduction in the PCr/ATP ratio in $Cx43^{Cre/fl}$ mice treated with 4-OHT, indicating impaired energetic metabolism in this group (Figure 26).

Table 4. Concentration of the intracellular metabolites analyzed in cardiac tissue extracts by ^{1}H-NMR spectroscopy (in µmol /10 µmol creatine).

	$Cx43^{fl/fl}$ + oil (n=8)	$Cx43^{fl/fl}$ + 4-OHT (n=8)	$Cx43^{Cre/fl}$ + oil (n=8)	$Cx43^{Cre/fl}$ + 4-OHT (n=8)
Total creatine (µmol / mg protein)	0.23 ± 0.03	0.21 ± 0.02	0.21 ± 0.03	0.16 ± 0.02
Lactate	7.31 ± 0.98	10.27 ± 1.23	9.31 ± 0.84	8.63 ± 0.60
Alanine	1.68 ± 0.10	1.85 ± 0.20	1.59 ± 0.08	1.69 ± 0.05
Succinate	0.85 ± 0.07	0.96 ± 0.11	0.81 ± 0.06	0.79 ± 0.11
Acetate	0.57 ± 0.11	0.65 ± 0.09	0.60 ± 0.04	0.72 ± 0.08
Taurine	20.45 ± 0.68	21.35 ± 1.21	20.77 ± 0.69	25.83 ± 1.35**
Formate	0.89 ± 0.06	0.65 ± 0.12	0.97 ± 0.18	1.02 ± 0.17
ATP	2.67 ± 0.44	2.45 ± 0.32	2.34 ± 0.33	2.74 ± 0.42

** ($p<0.01$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. all remaining groups.

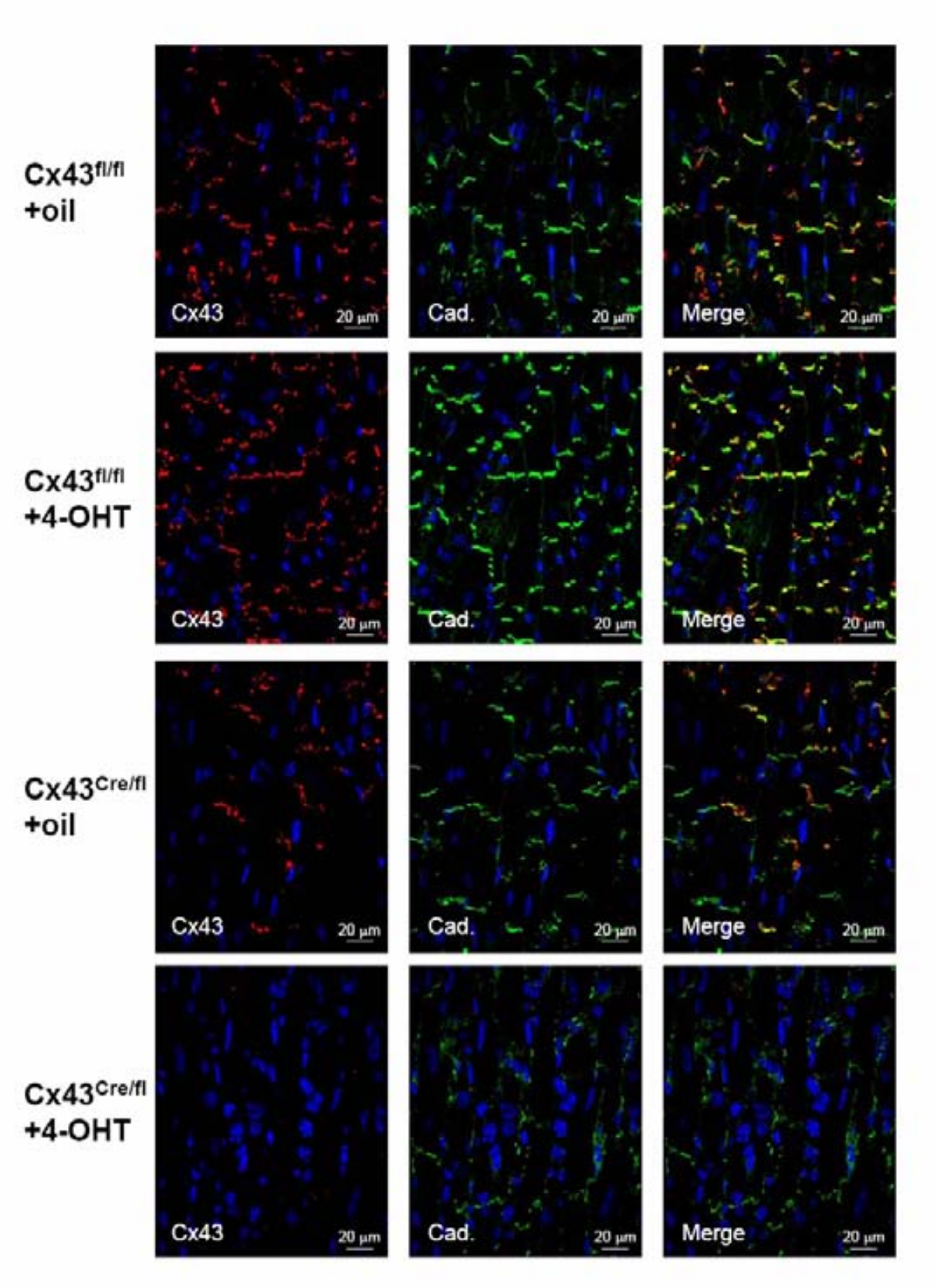


Figure 25.- Confocal images showing in red Cx43 expression in cardiac sections from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, either treated with vehicle or 4-OHT. Intercalated disks were marked with an antibody against pan-cadherin (Cad., green) and nuclei with Hoeschst 33342 (blue).

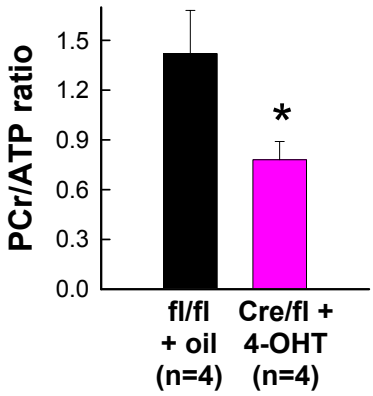


Figure 26.- PCr/ATP ratio measured by ^{31}P-NMR spectroscopy in isolated hearts from Cx43$^{fl/fl}$ mice treated with oil and Cx43$^{Cre/fl}$ mice treated with 4-OHT. * ($p<0.05$, Student's *t* test) indicates significant differences between both genotypes. Abbreviations as in Figure 24.

2.3- Effects of a marked reduction in Cx43 expression in Cx43$^{Cre-ER(T)/fl}$ mice on myocardial tolerance to ischemia-reperfusion injury

2.3.1- Left ventricular function

As occurred with Cx43KI32 mice, ischemic rigor contracture was detected as an abrupt increase in LVEDP during the first minutes of ischemia, and reperfusion was associated with a new increase in LVEDP, corresponding to hypercontracture. No differences in the time course of the changes in LVEDP were observed between the different groups (Figure 27A). However, maximal hypercontracture was significantly reduced in Cx43$^{Cre/fl}$ mice treated with 4-OHT, as compared with all remaining groups (Figure 27B). Functional recovery at the end of reperfusion was similar in all groups (Figure 27C), although hearts from Cx43$^{Cre/fl}$ mice treated with 4-OHT showed an initial rise in LVdevP, during the first three and four minutes of reperfusion, that was significantly higher than that observed in the remaining groups. Nevertheless, this initial improvement was rapidly lost due to the appearance of ventricular tachyarrhythmias.

2.3.2- Cell death

LDH release (Figure 28A and B) and infarct size (Figure 28C) were strongly reduced in isolated hearts from Cx43$^{Cre/fl}$ mice treated with 4-OHT, as compared with all other groups. No differences in both variables were observed between sexes in any genotype. However, and contrary to the findings observed in isolated hearts, protection

against ischemia-reperfusion injury was lost in isolated cardiomyocytes from these hearts. As can be seen in Figure 28D, cell death, assessed by Trypan blue staining, was similar in the four experimental groups after simulated ischemia-reperfusion.

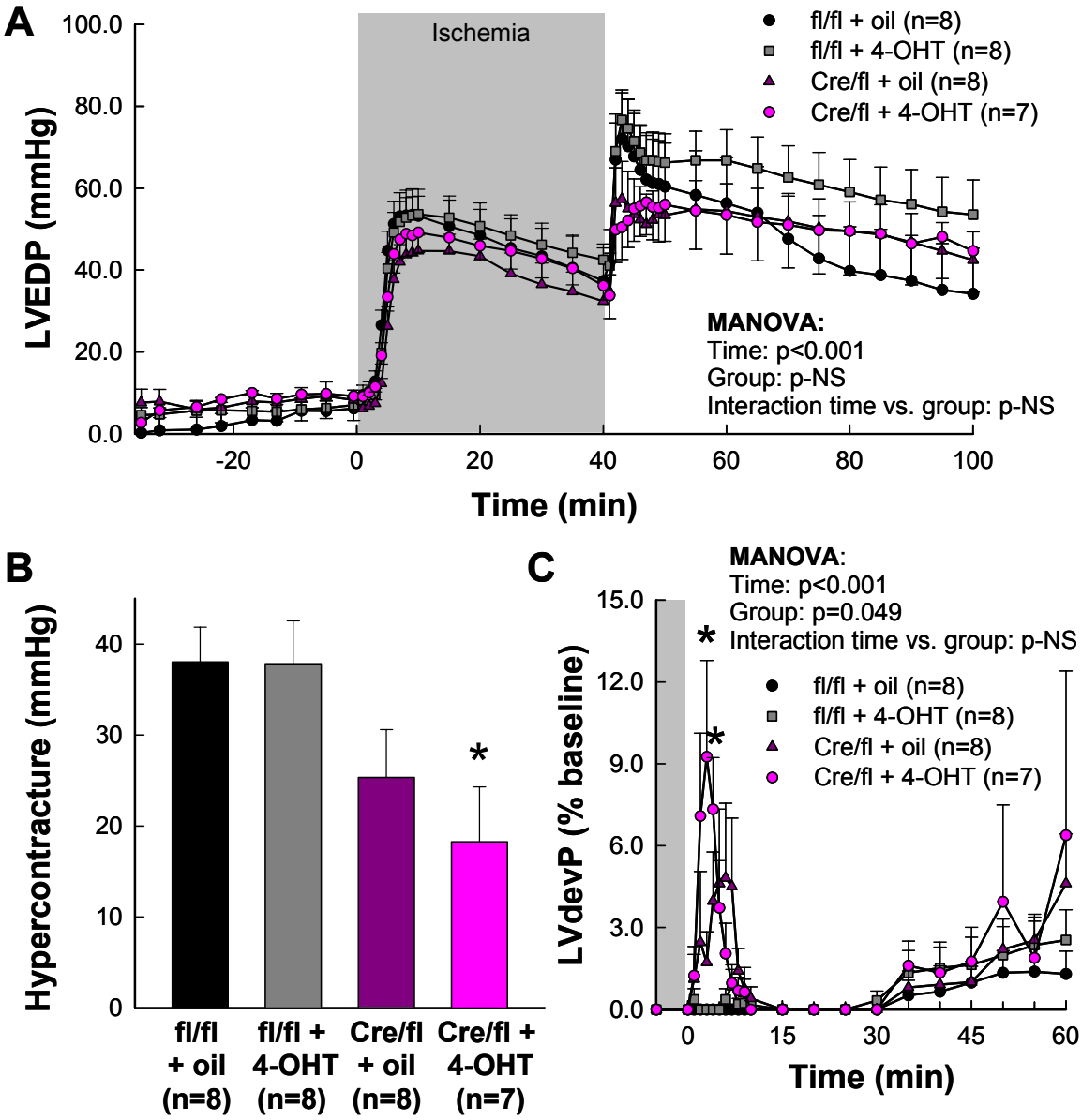


Figure 27.- (A) Changes in LVEDP observed in isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with oil or 4-OHT and submitted to 40 min of ischemia and 60 min of reperfusion. (B) Maximal hypercontracture, measured as the difference between peak LVEDP at the beginning of reperfusion and its last value during ischemia. * ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. results obtained in all remaining groups. (C) Functional recovery during reperfusion, expressed as percentage of baseline LVdevP in isolated hearts from the four experimental groups. * ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences vs. all remaining groups. Abbreviations as in Figure 24.

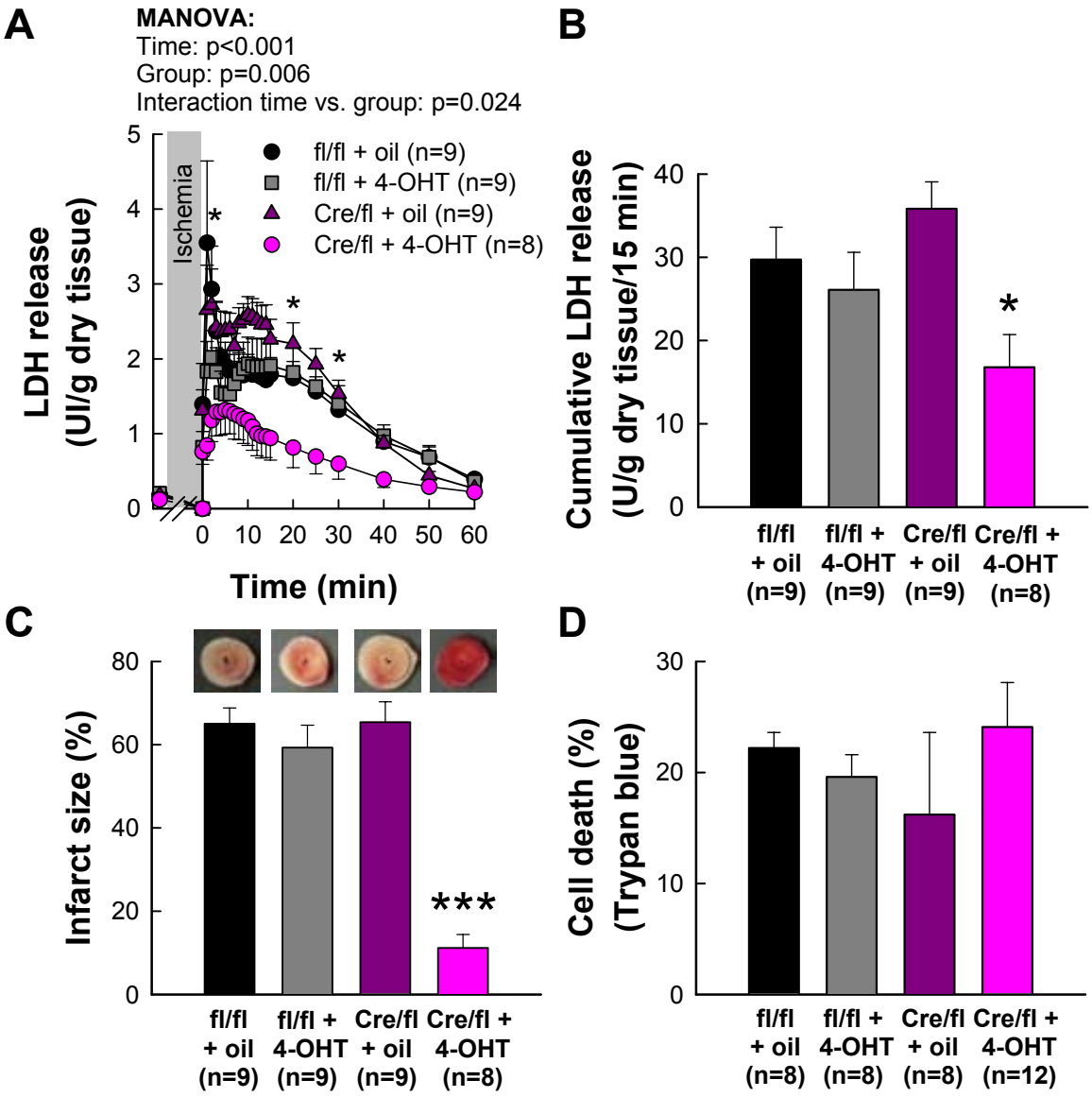


Figure 28.- LDH release during reperfusion (A) and its cumulative value during the first 15 minutes of flow restoration (B), determined in isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with oil or 4-OHT, and submitted to ischemia-reperfusion. * ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences between results obtained in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT and the remaining groups. (C) Infarct size, and representative images of cardiac slices stained with TTC, measured in hearts from the four experimental groups. (D) Cell death, analyzed by Trypan blue staining in isolated cardiomyocytes from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with oil or 4-OHT, and submitted to simulated ischemia-reperfusion. * ($p<0.05$) and *** ($p<0.001$) indicate significant differences vs. all remaining groups, as assessed by ANOVA and Tukey's *post hoc* tests. Abbreviations as in Figure 24.

2.3.3- Activation of cytosolic protective pathways during ischemia-reperfusion injury

The strong protection against ischemia-reperfusion injury observed in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT could hypothetically be due to an endogenous

baseline activation of cytosolic signaling cascades involved in protective pathways, as RISK and SAFE pathways. However, our western blot analysis demonstrated that the phosphorylated/ total protein ratio for Akt, ERK-1/2, GSK3β and STAT3 was not increased in animals lacking Cx43, as compared with the remaining groups (Figure 29). These data suggest that the increased tolerance against ischemia-reperfusion injury seen in isolated hearts from those animals is independent of a baseline activation of RISK and SAFE pathways.

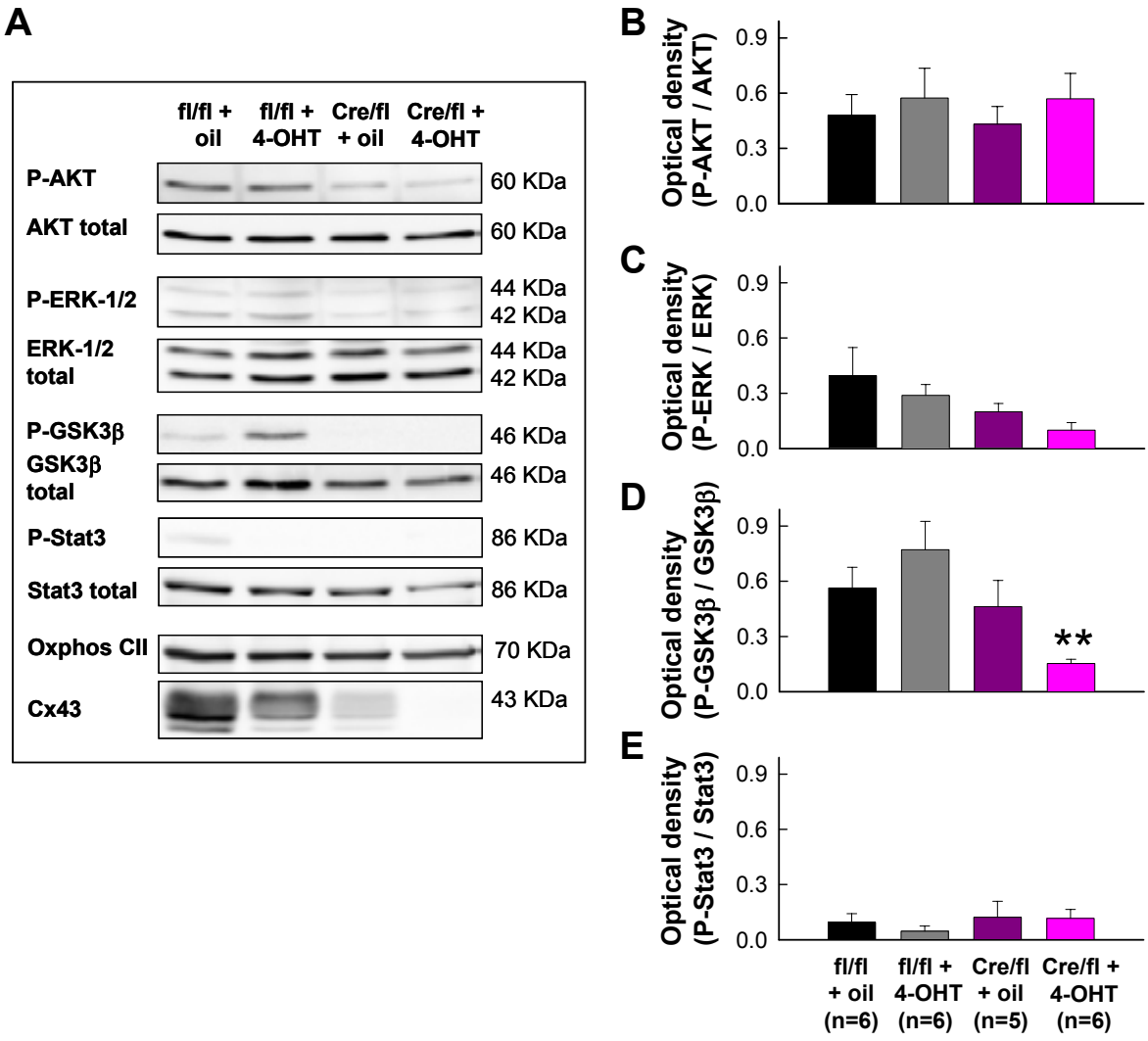


Figure 29.- (A) Representative western blots showing expression of the phosphorylated forms of Akt, ERK-1/2, GSk3β and STAT3, and compared with their total protein expression, in isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice treated with oil or 4-OHT, and perfused under normoxic conditions. Cx43 and Oxphos CII expression were also assessed in all experimental hearts. (B-E) Ratio between the optical density for the phosphorylated and total forms of Akt (B), ERK-1/2 (C), GSK3β (D), and STAT3 (E). ** ($p<0.01$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. results obtained in all remaining groups. Abbreviations as in Figure 24.

2.4- Effects of a marked reduction in Cx43 expression in Cx43$^{Cre-ER(T)/fl}$ mice on cardiac susceptibility to preconditioning protection

2.4.1- Left ventricular function and cell death

IPC significantly reduced hypercontracture (Figure 30A), and increased functional recovery during reperfusion (Figure 30B) in hearts from Cx43$^{fl/fl}$ mice treated with oil. Similar trends were observed in hearts from Cx43$^{fl/fl}$ mice treated with 4-OHT, and from Cx43$^{Cre/fl}$ animals treated with vehicle, but not in hearts from animals expressing very low levels of Cx43 (Cx43$^{Cre/fl}$ + 4-OHT).

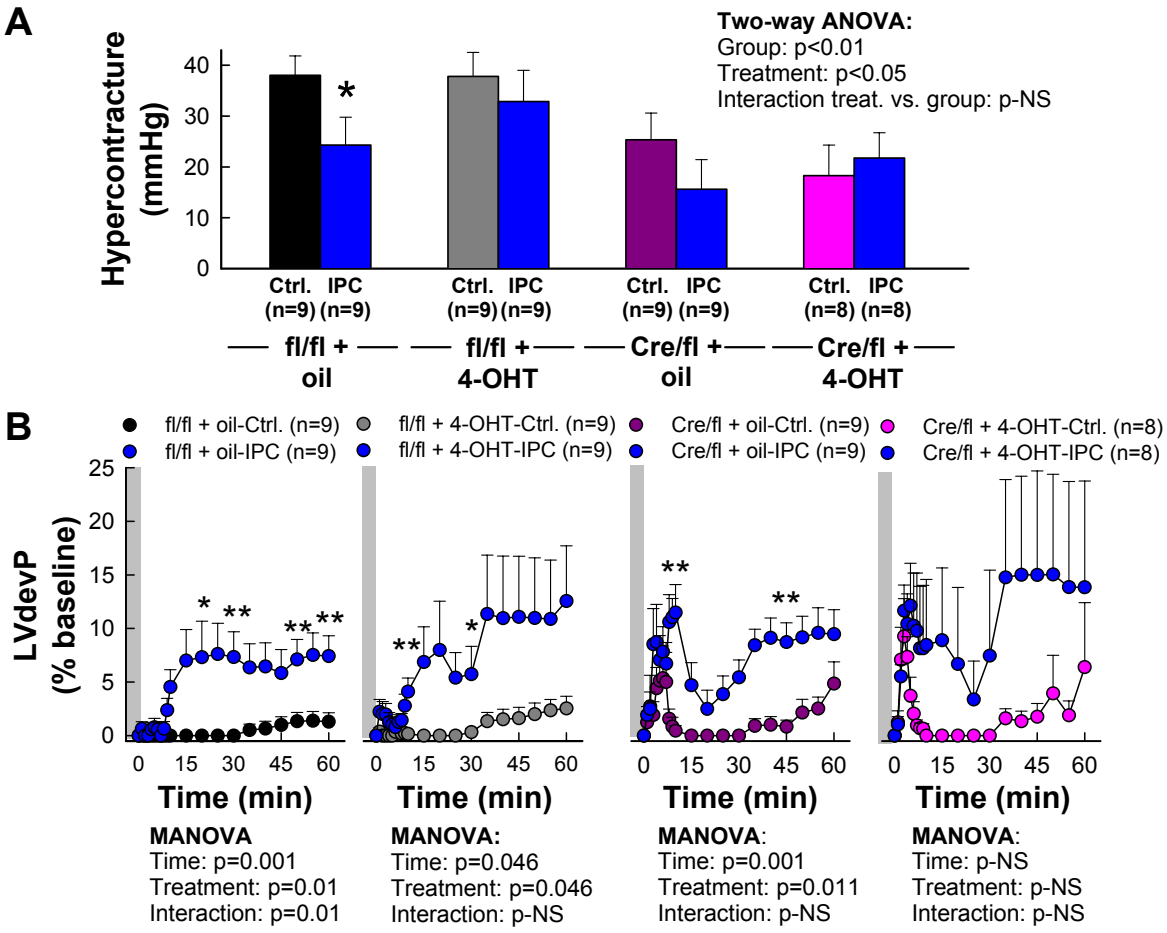


Figure 30.- (A) Maximal hypercontracture measured in isolated hearts from Cx43$^{fl/fl}$ and Cx43$^{Cre/fl}$ mice, treated with oil or 4-OHT, submitted to ischemia-reperfusion under control conditions (Ctrl.) or after IPC. (B) Functional recovery during reperfusion, calculated as a percentage of baseline LVdevP, in isolated hearts from the four experimental groups, under control conditions (Ctrl), or after IPC. * ($p<0.05$, Student's *t* test) indicates significant differences vs. the corresponding control. Abbreviations as in Figure 24.

Data on left ventricular function correlated with those obtained by LDH release and infarct size measurements. Thus, IPC was protective in all groups except in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT. This manoeuvre was able to significantly reduce LDH release (Figure 31A) and infarct size (Figure 31B) in hearts expressing a normal Cx43 content, or half of the normal content, but not in hearts having very low levels of Cx43 expression. However, this last result might be influenced by the very low degree of ischemia-reperfusion injury shown by control hearts from this group.

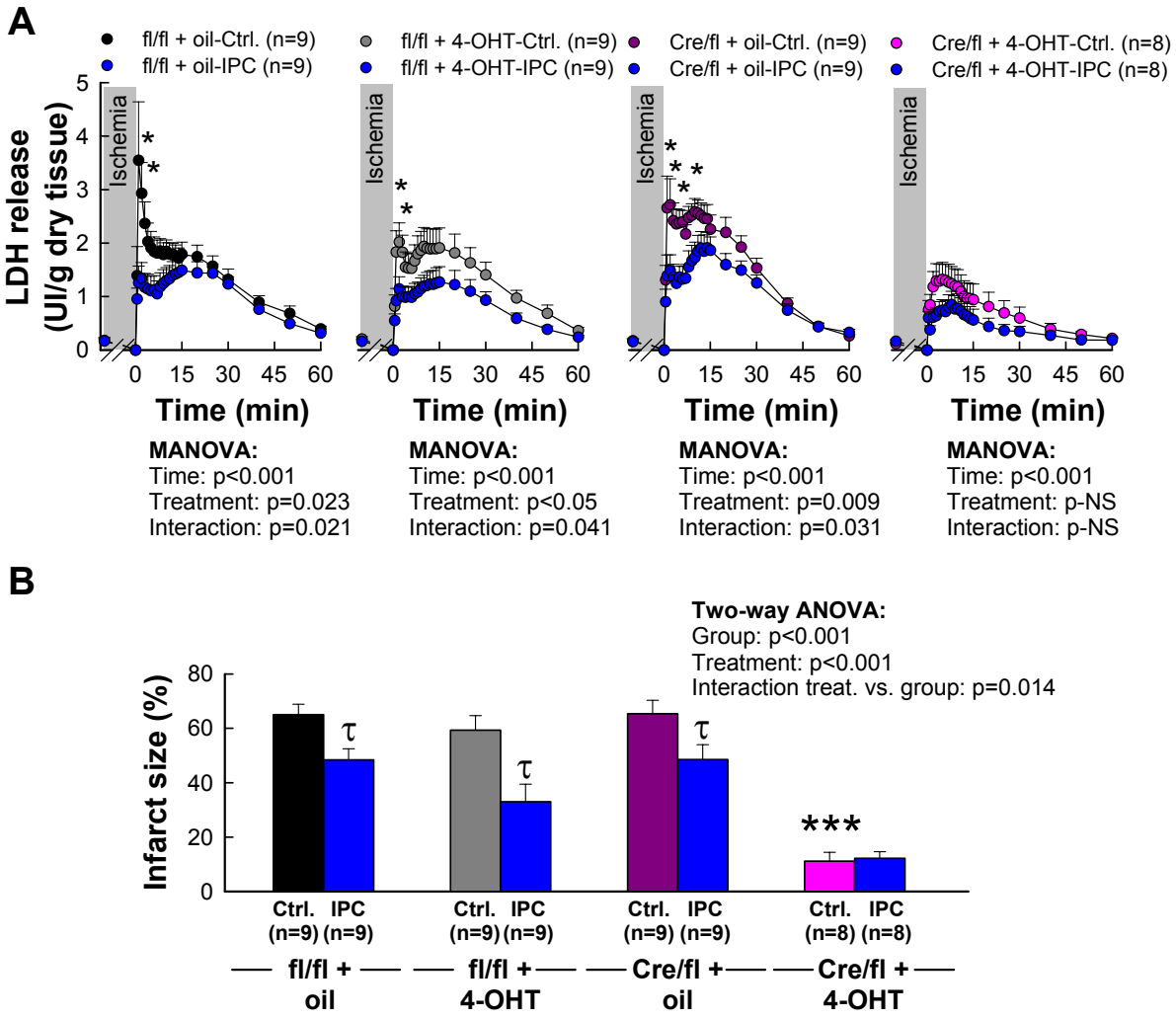


Figure 31.- (A) LDH release during reperfusion determined in isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with oil or 4-OHT, and submitted or not (Ctrl.) to IPC before ischemia-reperfusion. (B) Infarct size measured by TTC staining in the four experimental groups under control conditions (Ctrl.), or after IPC. * and τ ($p<0.05$) indicate significant differences vs. the corresponding controls, as assessed by Student's *t* test. *** ($p<0.001$, ANOVA and Tukey's *post hoc* tests) indicates significant differences between hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT and all remaining groups. Abbreviations as in Figure 24.

A two-way ANOVA test showed a significant effect of group ($Cx43^{fl/fl}$ or $Cx43^{Cre/fl}$ treated with oil or 4-OHT) and treatment (IPC or control) on hypercontracture and infarct size, indicating, respectively, an increased tolerance to myocardial ischemia-reperfusion injury in $Cx43^{Cre/fl}$ mice treated with 4-OHT, and protection by IPC in most of the groups. Furthermore, a significant interaction between group and treatment for infarct size was revealed by the two-way ANOVA analysis, suggesting that the degree of protection by IPC depends on the group, and reflects lack of protection in animals with very low levels of Cx43.

Previous studies in Cx43KI32 mice revealed that animals having 50% of normal Cx43 content lose protection, specifically, to pharmacological preconditioning with Dzx. As in our present model we were unable to see any effect of Cx43 deficiency on IPC protection, due to the low infarct size seen in this group lacking Cx43, we analyzed the effect of pharmacological preconditioning with Dzx on ischemia-reperfusion injury in hearts from $Cx43^{Cre/fl}$ mice treated with vehicle, expressing half of normal Cx43 content. As can be seen in figure 32, cell death, measured by LDH release (Figure 32A) and TTC staining (Figure 32B), was reduced after Dzx preconditioning in hearts from $Cx43^{fl/fl}$ mice treated with vehicle, but not in hearts from $Cx43^{Cre/fl}$ mice treated with oil, highlighting the importance of Cx43 for Dzx protection.

2.4.2- Activation of cytosolic protective pathways during IPC in animals with reduced Cx43 expression

Absence of protection by preconditioning in Cx43-deficient animals might be due to lack of activation of cytosolic RISK and SAFE protective signaling pathways. To analyze this possibility we tested, in extracts obtained from isolated mice hearts submitted to 4 cycles of transient ischemia-reperfusion, whether there is a differential activation of these pathways in Cx43-deficient animals. Our results demonstrated an increased phosphorylation of GSK3β and STAT3 in all hearts submitted to IPC (Figure 33), independently of group allocation, and a similar trend for ERK-1/2, suggesting that both RISK and SAFE pathways are equally activated independently of Cx43 expression.

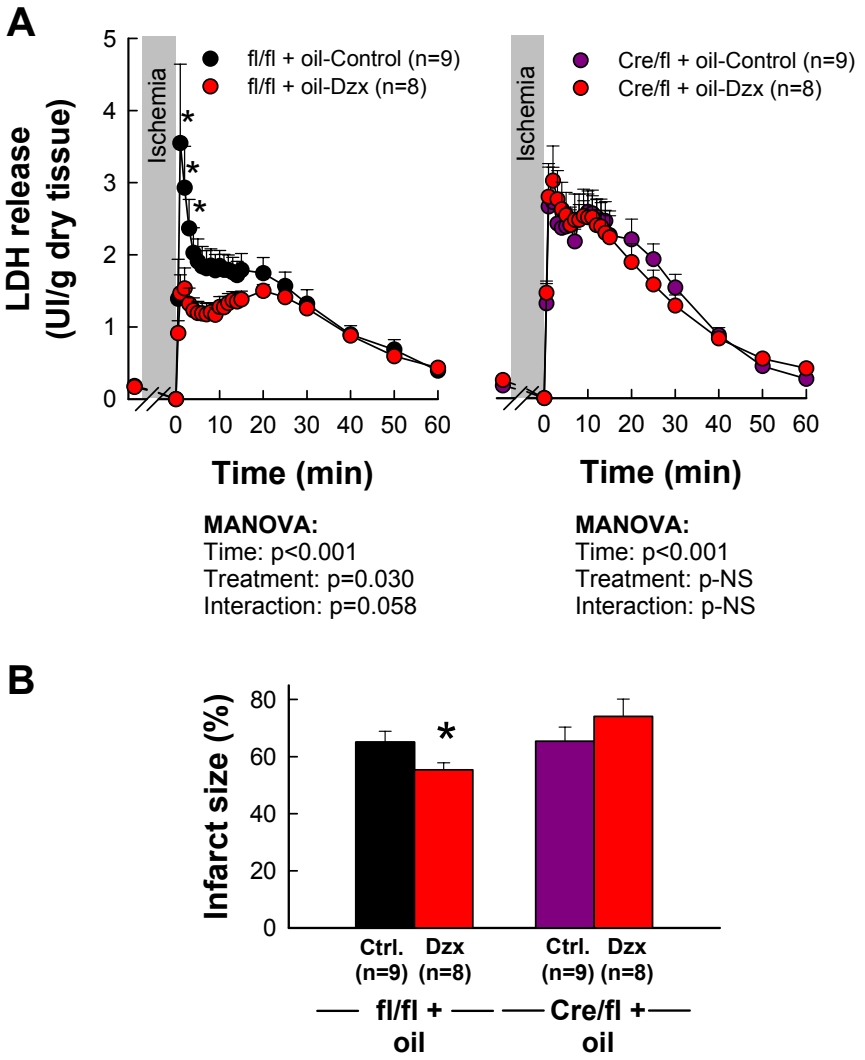


Figure 32.- (A) LDH release during reperfusion, evaluated in isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice treated with oil, and submitted to ischemia-reperfusion under control conditions (Ctrl.) or after preconditioning with Dzx. (B) Infarct size determined by TTC staining in these two genotypes, under control conditions (Ctrl.) or after treatment with Dzx. * ($p<0.05$, Student's *t* test) indicates significant differences vs. the corresponding control. Abbreviations as in Figure 24.

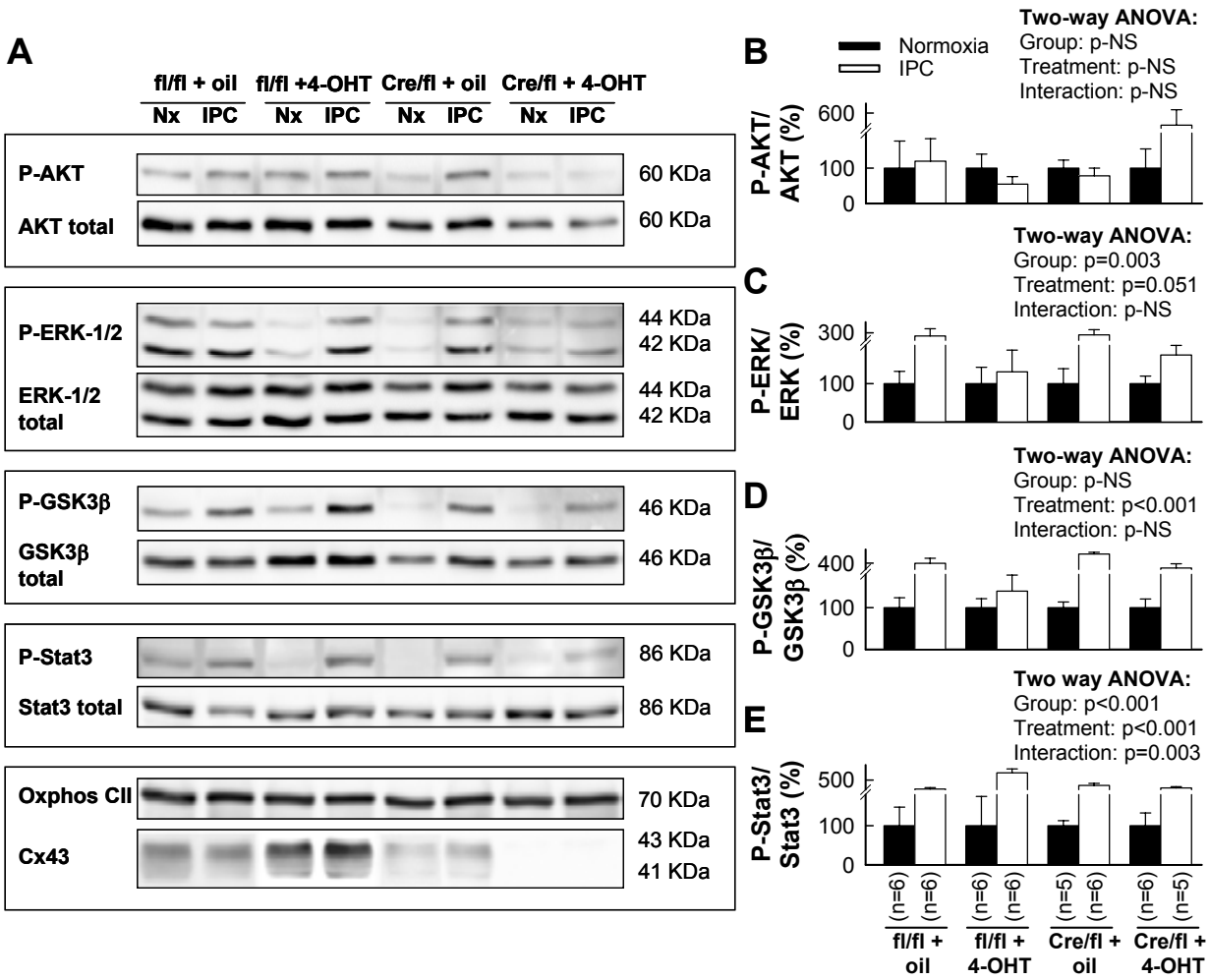


Figure 33.- (A) Representative western blots showing expression of the phosphorylated forms of AKT, ERK-1/2, GSK3β and STAT3, and compared with their total protein expression, in isolated hearts from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice, treated with oil or 4-OHT, and submitted to normoxic perfusion (Nx) or to IPC cycles. Cx43 and Oxphos CII expression were also assessed in all experimental hearts. (B-E) Ratio between the optical density for the phosphorylated and total forms of AKT (B), ERK-1/2 (C), GSK3β (D), and STAT3 (E), expressed as percentage of their normoxic value, in extracts from isolated hearts submitted to 34 minutes of normoxic perfusion or to IPC cycles. Abbreviations as in Figure 24.

3. Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on the incidence of ventricular arrhythmias, and on passive and active myocardial electrophysiological properties, during ischemia-reperfusion

3.1- Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on ventricular arrhythmogenesis

The effects of both a reduction in GJ unitary channel conductance or in the number of available GJ channels on ventricular arrhythmogenesis was studied, under normoxic conditions and during ischemia-reperfusion, in the two mice strains used in previous studies. Both genetic manipulations did not induce major changes in baseline heart rate, LVdevP or refractory period, except for an small increase in LVdevP in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT. In addition, area at risk, which was measured in these studies after regional ischemia and reperfusion, was similar in all groups (Table 5).

Table 5. Baseline values for heart rate, LVdevP, refractory period (also measured at minute 30 of reperfusion) and area at risk after regional ischemia-reperfusion in isolated hearts from Cx43KI32 mice and from $Cx43^{Cre\text{-}ER(T)/fl}$ animals treated with oil or 4-OHT.

	Heart rate (beats/min)	LVdevP (mmHg)	Refractory period (ms)		Area at risk (%)
			Baseline	Reperfusion	
Cx43KI32					
WT (n=15)	390.73±18.10	103.14±5.56	47.14±3.39	36.00±2.92	32.86±2.10
HOM (n=15)	391.03±15.26	92.64±7.97	39.64±1.92	32.00±1.22	39.19±3.31
$Cx43^{Cre\text{-}ER(T)/fl}$					
fl/fl+ oil (n=11)	398.33±9.15	95.24±5.39	46.36±3.57	47.00±6.82	28.34±2.25
fl/fl+ 4-OHT (n=10)	409.01±12.76	107.09±11.25	49.00±2.87	47.00±4.36	32.74±2.87
Cre/fl + oil (n=12)	387.03±9.91	86.16±7.19	41.25±1.75	42.50±3.59	27.56±1.46
Cre/fl + 4-OHT (n=11)	413.70±11.54	130.82±11.55 *	46.36±3.10	45.71±2.37	34.86±1.63

* ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. $Cx43^{fl/fl}$ mice treated with vehicle.

3.1.1- Incidence of spontaneous and inducible ventricular arrhythmias during normoxia

The number of spontaneous PVBs during 10 minutes of continuous pacing at BCL of 133 ms was significantly higher in isolated hearts from HOM Cx43KI32 mice and $Cx43^{Cre/fl}$ animals treated with 4-OHT, as compared with their corresponding genetic controls (WT and $Cx43^{fl/fl}$ treated with oil, respectively). Moreover, the number of spontaneous PVBs observed in isolated hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT was even higher than those found in hearts from HOM Cx43KI32 mice (Figure 34A and C). Differences in the number of spontaneous PVBs during 10 minutes of continuous pacing at BCL of 100 ms did not reach statistical significance (9.50 ± 3.23 in HOM vs. 3.29 ± 0.92 in WT, and 17.55 ± 5.85 in $Cx43^{Cre/fl}$ treated with 4-OHT vs. 5.45 ± 2.04 in $Cx43^{fl/fl}$ treated with oil).

Only few spontaneous ventricular tachyarrhythmias occurred during continuous pacing at BCL of 133 ms or 100 ms, with no differences between groups. In contrast, ventricular tachyarrhythmias could be induced by our protocol of programmed electrical stimulation described in material and methods. Ventricular tachyarrhythmias were induced often in hearts from HOM Cx43KI32 mice, and in those from $Cx43^{Cre/fl}$ animals treated with 4-OHT, compared with their corresponding controls (Figure 34B and D).

3.1.2- Incidence of spontaneous and inducible ventricular arrhythmias during regional ischemia-reperfusion

Regional ischemia increased the incidence of both spontaneous PVBs and ventricular tachyarrhythmias in isolated hearts from animals having normal Cx43 content. However, the number of both types of arrhythmias was even higher in hearts from animals with reduced GJ unitary channel conductance (HOM) and in those with a strong depletion in Cx43 content ($Cx43^{Cre/fl}$ mice treated with 4-OHT) (Figure 35A-D). Similar results were observed during reperfusion in HOM mice, but not in $Cx43^{Cre/fl}$ mice treated with 4-OHT, although they showed a non significant trend towards an increased spontaneous ventricular arrhythmogenesis during this phase (Figure 35E-F).

Effective refractory periods were not significantly modified during ischemia (Figure 36) or reperfusion (Table 5) in isolated hearts from WT mice and from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ animals, treated either with 4-OHT or vehicle. In contrast, ischemia induced a moderate increase in effective refractory periods in hearts from HOM

Cx43KI32 mice, reaching a maximum between 30 and 45 minutes of ischemia (Figure 36).

Programmed electrical stimulation during regional ischemia was associated with an enhanced inducibility of ventricular tachyarrhythmias in hearts from HOM Cx43KI32 mice and from $Cx43^{Cre/fl}$ animals treated with 4-OHT, compared with remaining groups (Figure 37A and B). Moreover, their mean duration was also longer in both transgenic models of Cx43 depletion (Figure 37C).

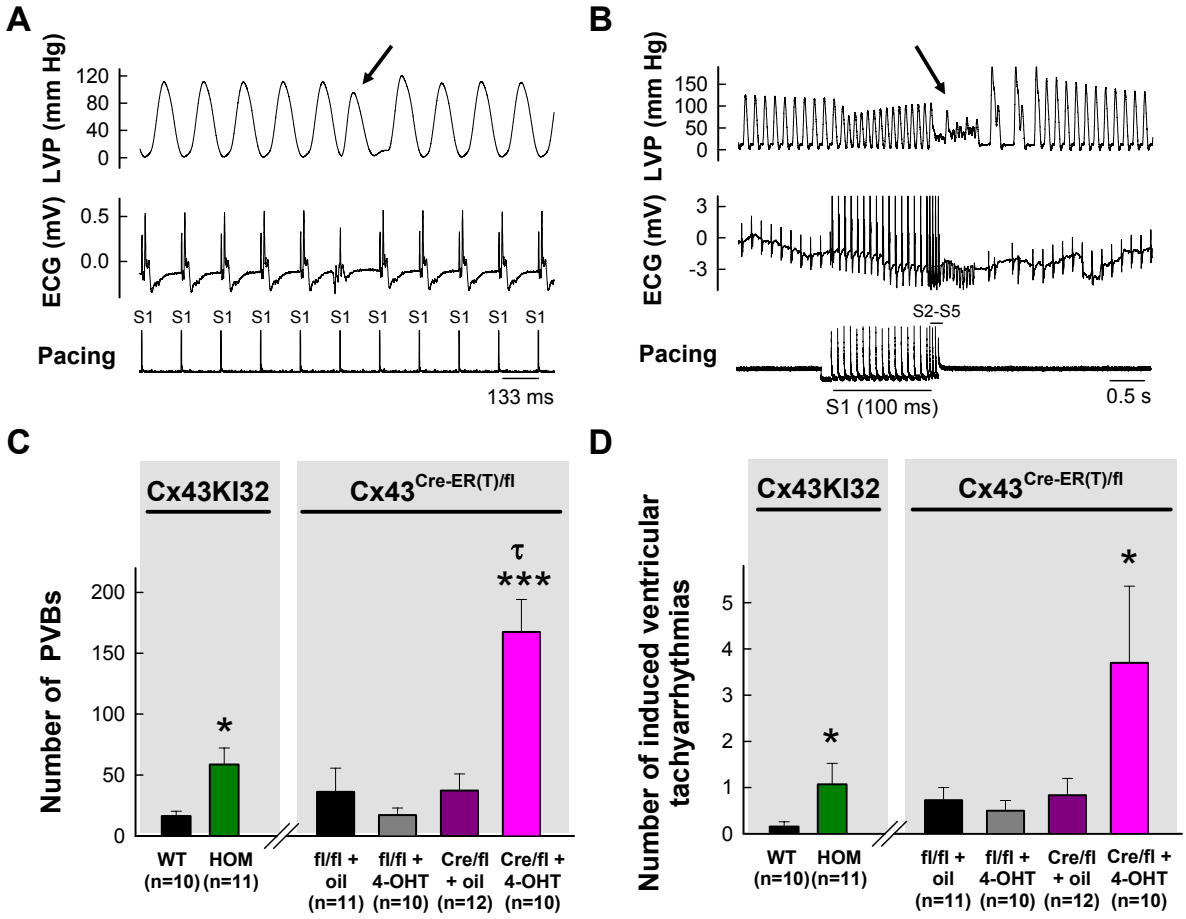


Figure 34.- (A and B) Representative recordings of a spontaneous PVB in an isolated heart from a HOM Cx43KI32 mouse during 10 minutes of continuous pacing at BCL of 133 ms (A), and of a short run of ventricular tachyarrhythmia induced by 4 extrastimuli applied after a train of 16 beats at BCL of 100 ms in an isolated heart from a $Cx43^{Cre/fl}$ mice treated with 4-OHT (B). Arrows show the corresponding arrhythmia. (C) Number of spontaneous PVBs observed during 10 minutes of continuous pacing at BCL of 133 ms. (D) Number of ventricular tachyarrhythmias induced by the protocol of programmed electrical stimulation. * ($p<0.05$) and *** ($p<0.001$) indicate significant differences vs. the corresponding controls (WT or $Cx43^{fl/fl}$ mice treated with oil), as assessed by Mann Whitney-U (Cx43KI32 mice) or Kruskal-Wallis tests ($Cx43^{Cre\text{-}ER(T)/fl}$ animals). τ ($p<0.001$, Kruskal-Wallis test) indicates significant differences between hearts from HOM Cx43KI32 mice and $Cx43^{Cre/fl}$ animals treated with 4-OHT. Abbreviations as in Figure 24.

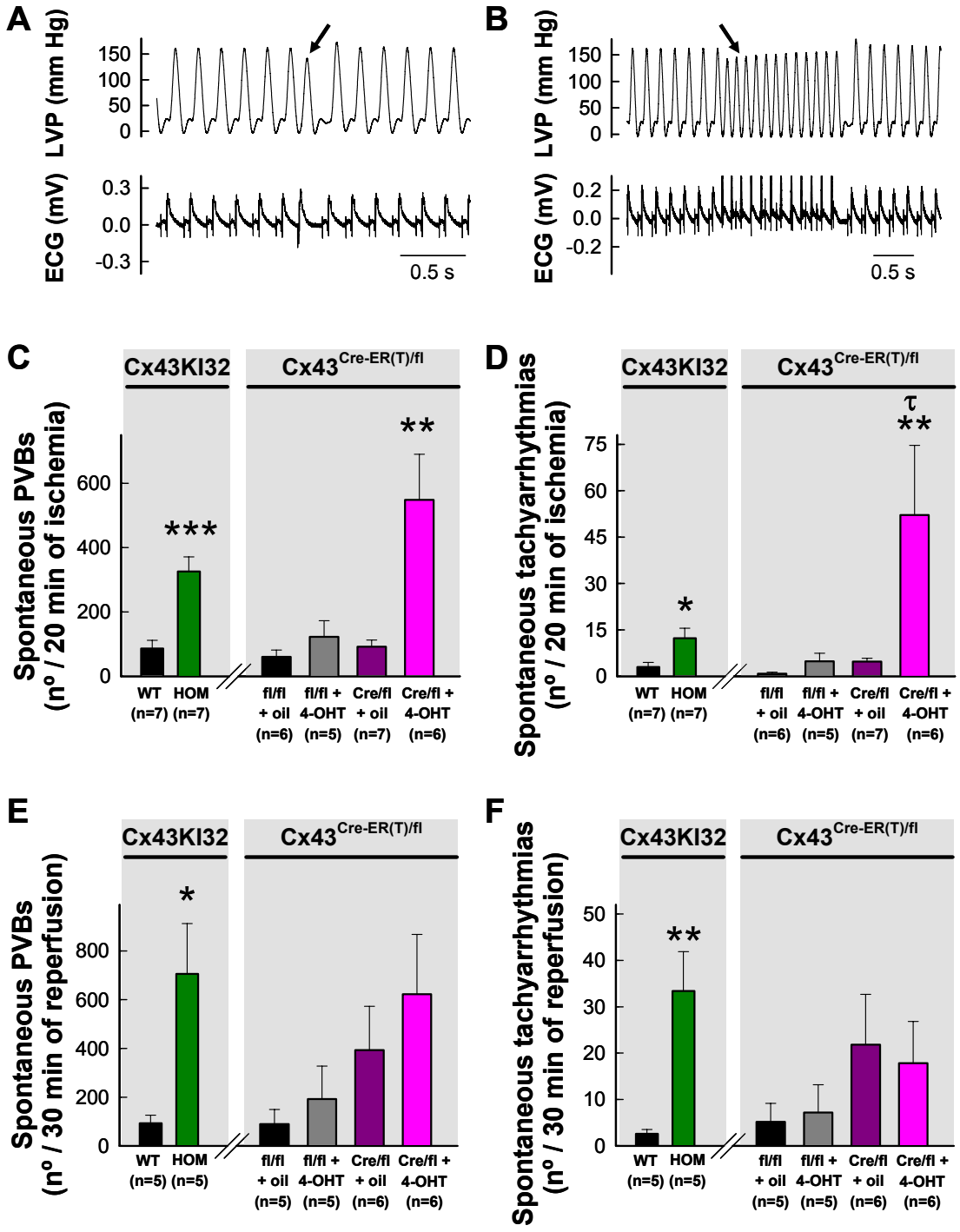


Figure 35.- (A and B) Representative recordings showing a spontaneous PVB (A) and a spontaneous ventricular tachyarrhythmia (B) occurring during regional ischemia in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT. Arrows indicate the corresponding arrhythmia. (C and D) Total number of spontaneous PVBs (C) and spontaneous ventricular tachyarrhythmias (D) during 20 minutes of regional ischemia. (E and F) Total number of spontaneous PVBs (E) and spontaneous ventricular tachyarrhythmias (F) observed during 30 minutes of reperfusion after regional ischemia. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences vs. the corresponding controls (WT or $Cx43^{fl/fl}$ mice treated with oil), as assessed by Mann Whitney-U (Cx43KI32 mice) or Kruskal-Wallis tests ($Cx43^{Cre-ER(T)/fl}$ animals). τ ($p<0.05$, Kruskal-Wallis test) indicates significant differences between hearts from HOM Cx43KI32 mice and $Cx43^{Cre/fl}$ animals treated with 4-OHT. Abbreviations as in Figure 24.

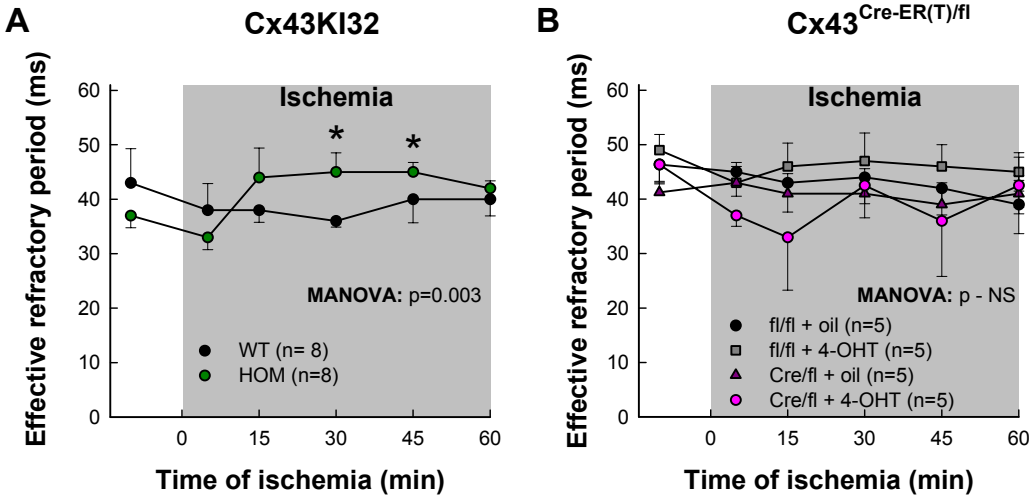


Figure 36.- Changes in effective refractory periods observed during regional ischemia in isolated hearts from Cx43KI32 mice (A) and $Cx43^{Cre\text{-}ER(T)/fl}$ animals (B). * ($p<0.05$, Student's *t* test) indicates significant differences between hearts from WT and HOM Cx43KI32 mice. Abbreviations as in Figure 24.

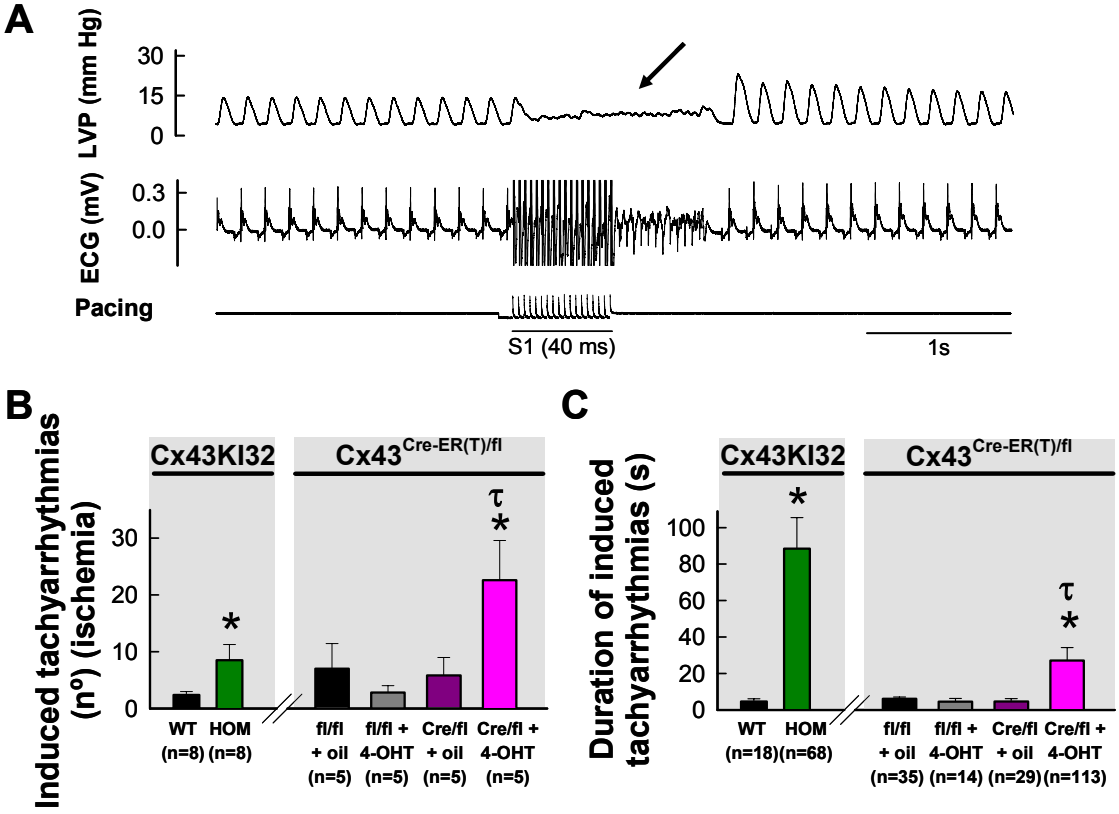


Figure 37.- (A) Representative recording of a short run of ventricular tachyarrhythmia induced during ischemia (arrow) by burst pacing at BCL of 40 ms in a heart from a HOM Cx43KI32 mouse. (B) Total number of ventricular tachyarrhythmias induced by programmed electrical stimulation during 60 minutes of regional ischemia, and their mean duration (C), in isolated hearts from Cx43KI32 mice and $Cx43^{Cre\text{-}ER(T)/fl}$ animals. * ($p<0.05$) indicates significant differences vs. the corresponding controls (WT and $Cx43^{fl/fl}$ treated with oil), as assessed by Mann Whitney-U (Cx43KI32 mice) or Kruskal-Wallis tests ($Cx43^{Cre\text{-}ER(T)/fl}$ animals). τ ($p<0.05$, Kruskal-Wallis test) indicates significant differences between hearts from HOM Cx43KI32 mice and $Cx43^{Cre/fl}$ animals treated with 4-OHT. Abbreviations as in Figure 24.

During reperfusion, a trend was observed towards an increased number of induced ventricular tachyarrhythmias in isolated hearts from HOM Cx43KI32 mice and from $Cx43^{Cre/fl}$ animals treated with 4-OHT, that, however, did not reach statistical significance (Figure 38A and B). In contrast, their mean duration was significantly longer in both transgenic models of Cx43 deficiency (Figure 38C).

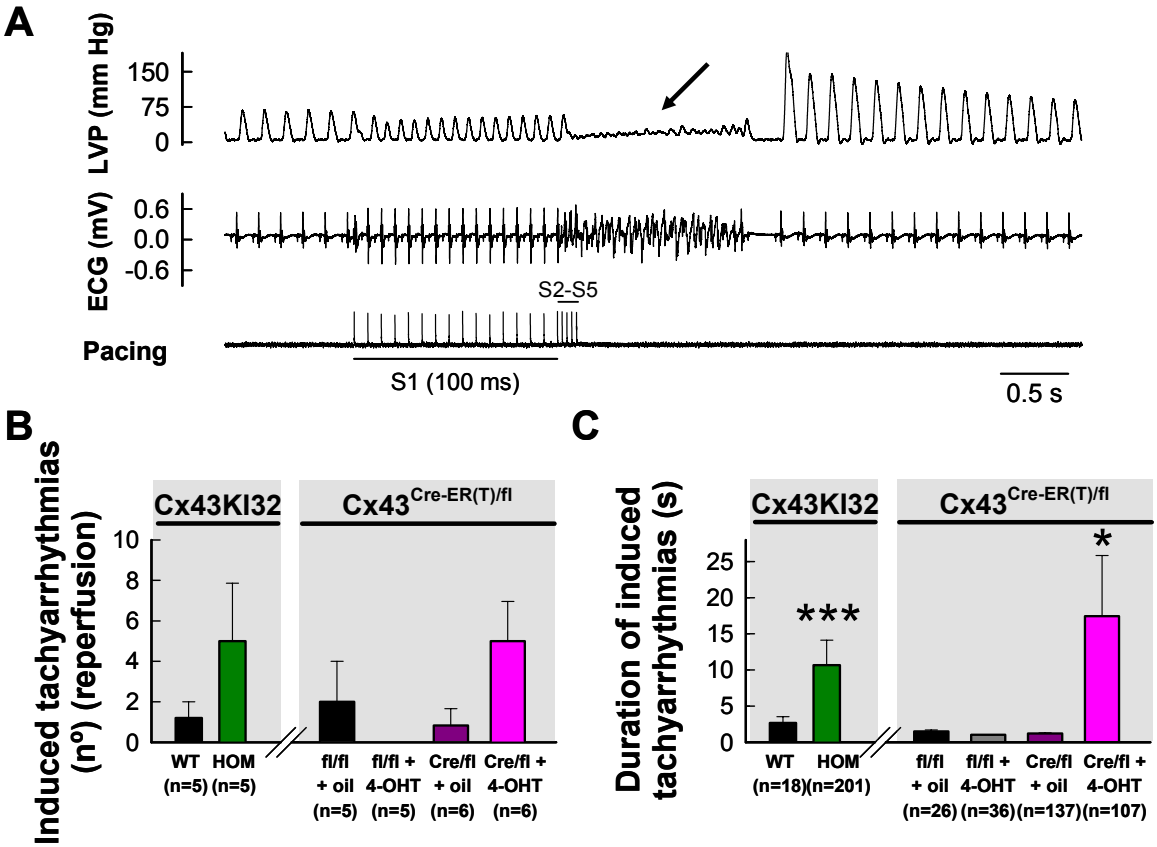


Figure 38.- (A) Representative recording showing a short run of ventricular tachyarrhythmia induced during reperfusion (arrow), by four extrastimuli in an isolated heart from a HOM Cx43KI32 mouse. (B) Total number of ventricular tachyarrhythmias induced by programmed electrical stimulation 30 minutes after reperfusion, and their mean duration (C), in isolated hearts from Cx43KI32 mice and $Cx43^{Cre-ER(T)/fl}$ animals. * ($p<0.05$) and *** ($p<0.001$) indicate significant differences vs. the corresponding controls (WT and $Cx43^{fl/fl}$ treated with oil), as assessed by Mann Whitney-U (Cx43KI32 mice) or Kruskal-Wallis tests ($Cx43^{Cre-ER(T)/fl}$ animals). Abbreviations as in Figure 24.

This increased ventricular arrhythmogenesis observed during ischemia-reperfusion in isolated hearts from animals having reduced GJ unitary channel conductance, and from those with a marked reduction in the number of available GJ channels, could not be attributed to differences in the ligature location, as the size of

area at risk was similar in all experimental groups (Table 5). However, infarct size, calculated as a percentage of area at risk after 20 minutes of regional ischemia and 60 minutes of reperfusion, was significantly reduced in hearts from HOM Cx43KI32 mice and from $Cx43^{Cre/fl}$ animals treated with 4-OHT, compared with their corresponding controls (Figure 39).

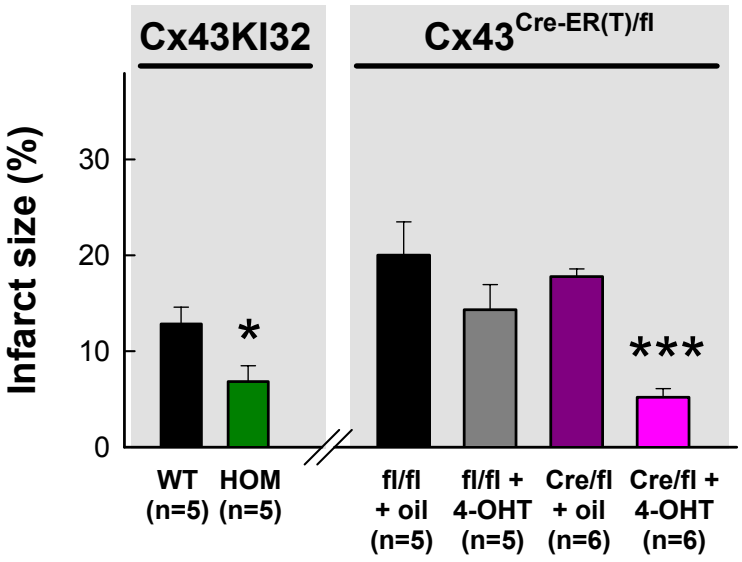


Figure 39.- Infarct size, measured by TTC staining and expressed as percentage of area at risk, in isolated hearts from Cx43KI32 and $Cx43^{Cre\text{-}ER(T)/fl}$ mice, submitted to 20 minutes of regional ischemia and 60 minutes of reperfusion. * ($p<0.05$, Student's *t* test) and *** ($p<0.001$, ANOVA and Tukey's *post hoc* tests) indicate significant differences vs. the corresponding controls (WT and $Cx43^{fl/fl}$ treated with oil). Abbreviations as in Figure 24.

3.2- Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on passive and active myocardial electrical properties

3.2.1- Conduction velocity

Replacement of Cx43 by Cx32 did not modify baseline conduction velocity in isolated hearts from Cx43KI32 mice (Table 6 and Figure 40 C). In contrast, treatment with 4-OHT in $Cx43^{Cre/fl}$ mice, inducing a severe Cx43 depletion, significantly reduced conduction velocity under baseline conditions to about half of that recorded in the corresponding control hearts (Table 6 and Figure 40 D). During global ischemia,

conduction was markedly impaired in all groups, which was reflected as a steep reduction in conduction velocity (Figure 40). However, such impairment was significantly more pronounced in isolated hearts from HOM Cx43KI32 mice, compared with those from WT animals, but not in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT (Figure 40). Conduction blockade occurred significantly earlier in hearts from $Cx43^{Cre/fl}$ animals treated with 4-OHT (Table 6 and Figure 40).

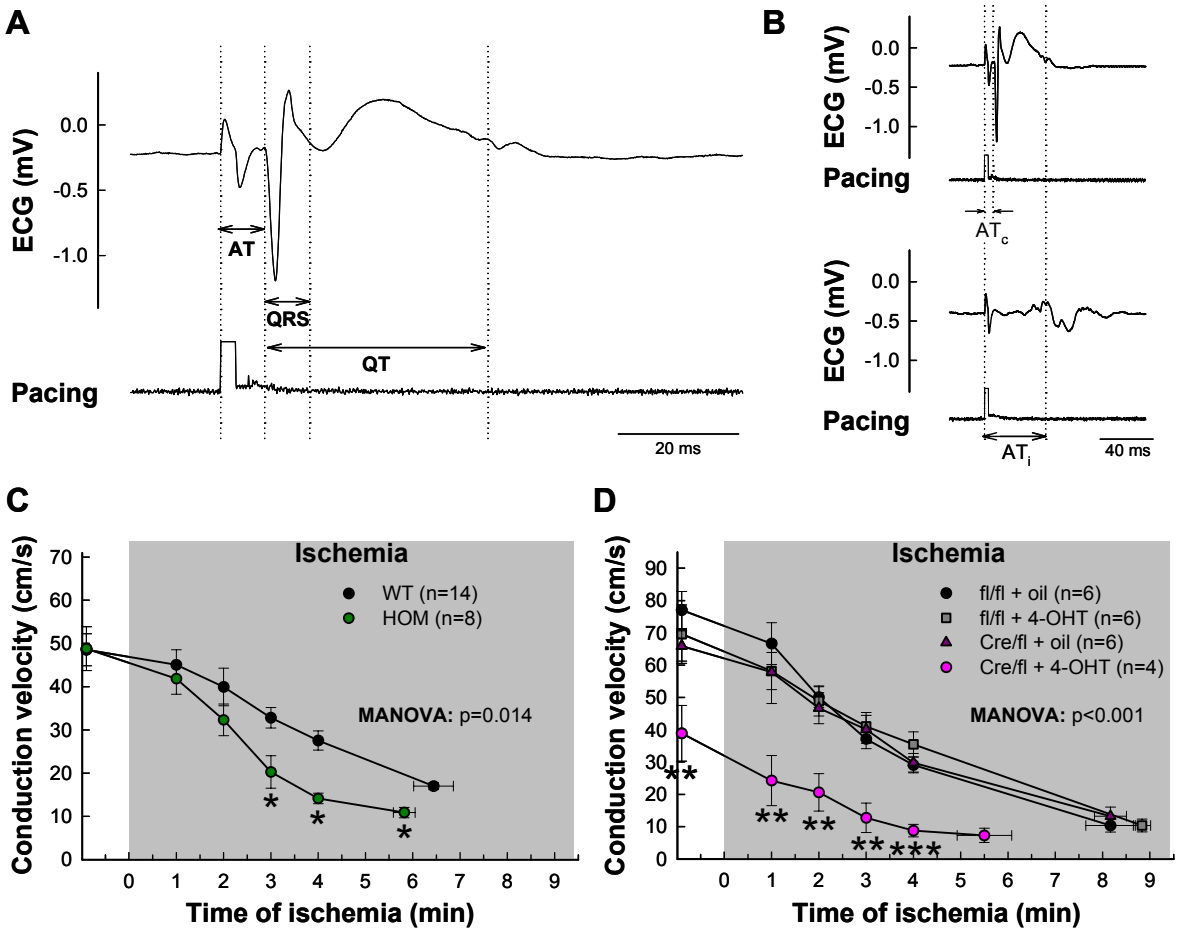


Figure 40.- (A and B) High-resolution electrograms showing the method of measurement of activation times in isolated mice hearts (A), and how activation time was prolonged after 6 minutes of global ischemia in an isolated heart from a $Cx43^{fl/fl}$ mouse treated with vehicle. (C and D) Changes in conduction velocity during 40 minutes of global ischemia in isolated hearts from Cx43KI32 mice (C) and from $Cx43^{Cre\text{-}ER(T)/fl}$ animals (D). * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) indicates significant differences vs. the corresponding controls (WT and $Cx43^{fl/fl}$ treated with oil), as assessed by MANOVA and Dunnett's *post hoc* tests. Abbreviations: AT: activation time, AT_c and AT_i: activation time under control and ischemic conditions, respectively. Other abbreviations as in Figure 24.

3.2.2- Passive myocardial electrical properties: tissue resistivity

Replacement of Cx43 by Cx32 in the Cx43KI32 model, and the strong depletion of Cx43 expression in 4-OHT treated $Cx43^{Cre/fl}$ mice, did not modify baseline myocardial electrical impedance (Table 6). Ischemia induced a marked increase in myocardial resistivity and a decrease in phase angle, which quickly recovered upon reperfusion (Figure 41). However, the maximal change in tissue resistivity induced by ischemia was significantly lower in hearts from HOM Cx43KI32 mice, as compared with hearts from WT animals. Moreover, changes in both tissue resistivity and phase angle were also significantly attenuated in hearts from $Cx43^{Cre/fl}$ mice, especially after 4-OHT treatment (Figure 41). In contrast, the onset of the sharp changes in myocardial resistivity and phase angle, which has been related to the onset of cell-to-cell electrical uncoupling, was similar in all groups, both determined in resistivity or phase angle recordings (Table 6).

Table 6. Baseline values for tissue resistivity and phase angle, conduction velocity, time of conduction blockade, and onset of the sharp changes in electrical impedance during ischemia in isolated hearts from Cx43KI32 and $Cx43^{Cre\text{-}ER(T)/fl}$ mice treated with oil or 4-OHT.

	Baseline conduction velocity, cm/s	Conduction blockade, min	Baseline tissue resistivity, Ω·cm	Baseline phase angle, °	Sharp changes in tissue resistivity, min	Sharp changes in phase angle, min
Cx43KI32						
WT (n=8-14)	48.54±3.71	6.44±0.42	126.89±13.16	-1.76±0.12	3.10±0.22	3.23±0.26
HOM (n=8-9)	48.80±5.07	5.82±0.23	133.77±23.29	-1.66±0.11	3.25±0.22	3.46±0.21
$Cx43^{Cre\text{-}ER(T)/fl}$						
fl/fl+ oil (n=5-6)	77.04±5.75	8.17±0.52	202.72±19.17	-1.77±0.29	6.58±0.58	6.13±0.53
fl/fl+ 4-OHT (n=5-6)	69.61±9.06	8.53±0.18	141.92±18.69	-1.72±0.32	7.21±0.37	6.79±0.14
Cre/fl + oil (n=5-6)	65.91±4.67	8.17±0.34	176.72±39.98	-1.52±0.40	6.50±0.32	6.58±0.20
Cre/fl + 4-OHT (n=5-6)	38.90±8.61 **	5.50±0.58 *	198.92±44.77	-1.20±0.25	5.58±0.44	5.50±0.77

* ($p<0.05$) and ** ($p<0.01$) indicate significant differences vs. $Cx43^{fl/fl}$ mice treated with oil, as assessed by ANOVA and Tukey's *post hoc* tests.

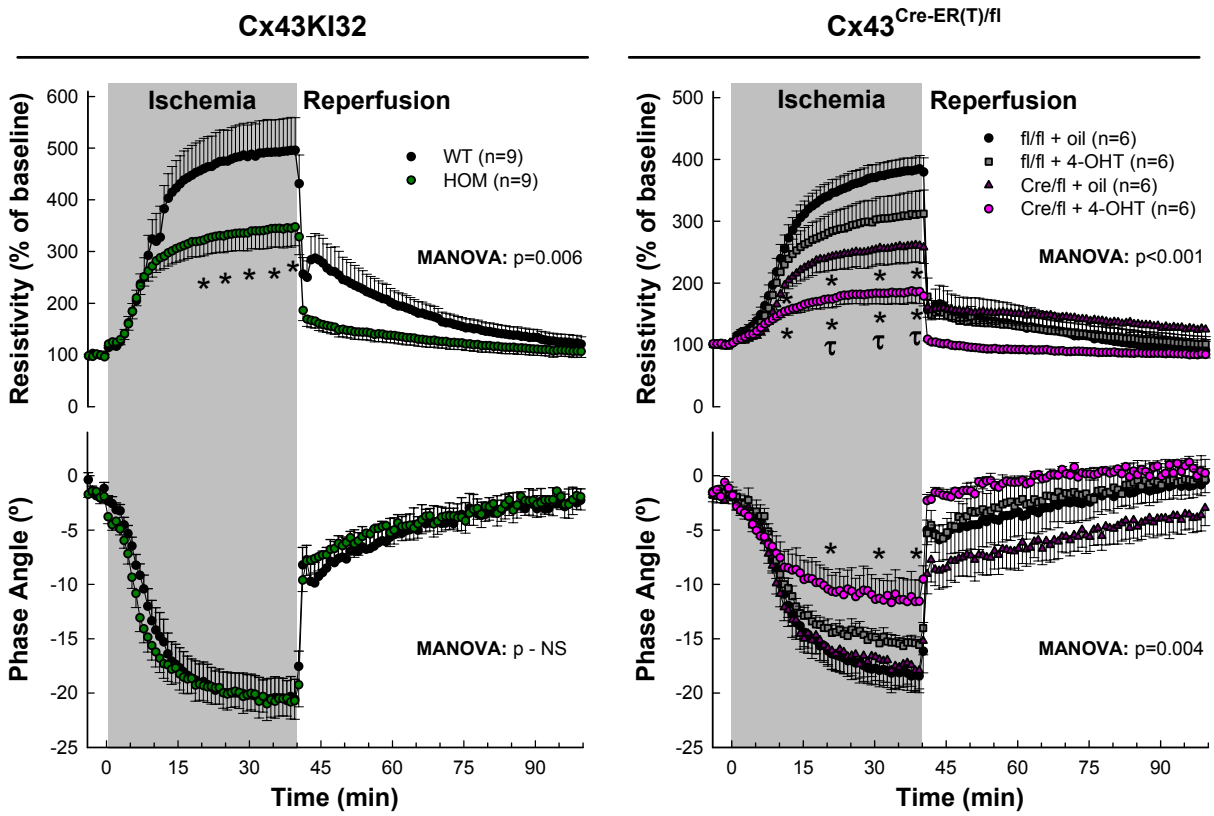


Figure 41.- Changes in myocardial electrical impedance (tissue resistivity (top) and phase angle (bottom)), during 40 minutes of global ischemia and 60 minutes of reperfusion in isolated hearts from Cx43KI32 mice (left) and $Cx43^{Cre\text{-}ER(T)/fl}$ animals (right). * ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences vs. the corresponding controls (WT and $Cx43^{fl/fl}$ treated with oil). τ ($p<0.05$, MANOVA and Dunnett's *post hoc* tests) indicates significant differences between hearts from $Cx43^{Cre/fl}$ animals treated with 4-OHT and oil. Abbreviations as in Figure 24.

3.2.3- Active myocardial electrical properties: transmembrane action potentials

The increased ventricular arrhythmogenesis seen in hearts from HOM Cx43KI32 mice cannot be ascribed to differences in action potential characteristics, as all parameters were similar to those observed in hearts from WT animals (Table 7 and Figure 42). In contrast, hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT showed a significant increase in dV/dt_{max} values, and a prolongation in action potential duration, compared with hearts from $Cx43^{fl/fl}$ animals treated with vehicle (Table 7 and Figure 42).

Table 7. Transmembrane action potential parameters recorded using floating intracellular microelectrodes in isolated hearts from Cx43KI32 animals, and $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ mice treated with oil or 4-OHT, respectively. Measurements were made during continuous pacing at BCL of 133 ms and in the presence of blebbistatin 10 μM.

	Cx43KI32 mice		**$Cx43^{Cre\text{-}ER(T)/fl}$**	
	WT	HOM	fl/fl+ oil	Cre/fl + 4-OHT
Resting membrane potential, mV	-83.53±0.95	-81.16±1.54	-84.02±0.48	-82.01±0.58
AP amplitude, mV	96.65±0.99	94.98±1.49	97.52±1.23	101.85±2.41
dV/dt_{max}, V/s	261.60±14.77	267.61±5.67	212.53±4.48	280.33±11.79*
APD_{25}, ms	4.40±0.13	4.90±0.22	5.30±0.19	6.20±0.24 *
APD_{50}, ms	9.20±0.28	9.90±0.42	11.00±0.24	14.80±0.68 *
APD_{75}, ms	26.40±0.58	25.60±1.22	31.10±0.99	37.30±1.87 *

Values are mean ± SEM; n=3 animals/group (5 different impalements/heart). * ($p<0.05$, ANOVA and Tukey's *post hoc* tests) indicates significant differences vs. $Cx43^{fl/fl}$ mice treated with vehicle. Abbreviations: AP: action potential, APD25, APD50 and APD75: action potential duration at 25%, 50% and 75% repolarization. Other abbreviations as in Figure 24.

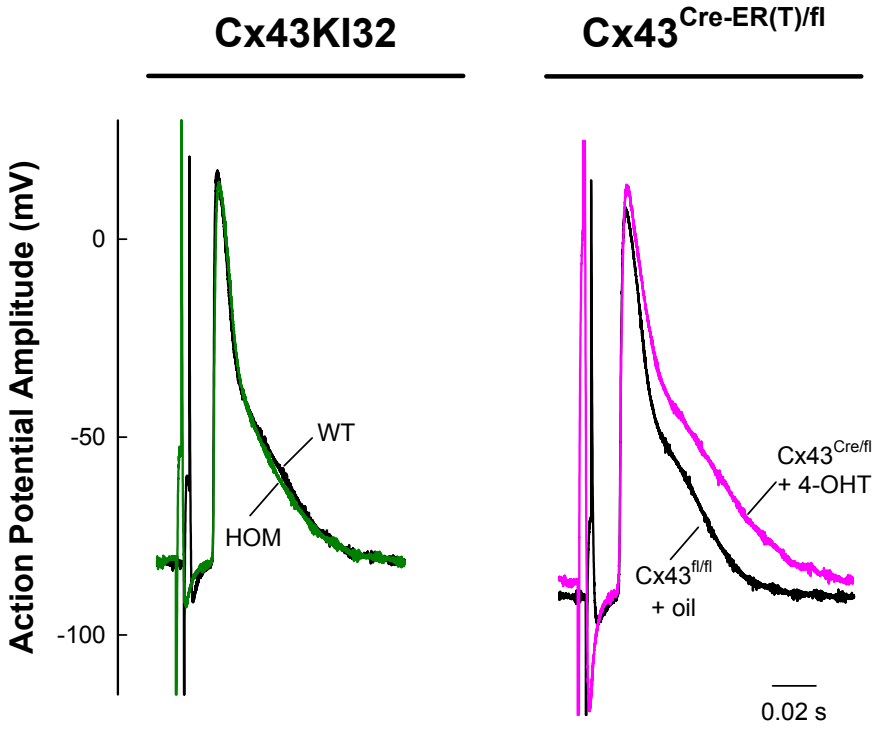


Figure 42.- Transmembrane action potentials recorded at BCL of 133 ms in isolated hearts from Cx43KI32 mice (left), and from $Cx43^{fl/fl}$ and $Cx43^{Cre/fl}$ animals treated with oil and 4-OHT, respectively (right). Recordings were obtained in the presence of 10 μM blebbistatin.

Discussion

1. Effects of replacement of Cx43 by Cx32 in Cx43KI32 mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

Replacement of Cx43 by Cx32 in Cx43KI32 mice mimics a situation of reduced maximal unitary conductance of connexin channels. Cx32, the major GJ protein in the liver, is also expressed in other tissues, including neurons and oligodendrocytes, vascular endothelial cells and tumours, but not in myocardium, and shows a reduced electrical conductivity and chemical permeability to several ions and intracellular messengers (Niessen et al., 2000; Goldberg et al., 2002), as compared with Cx43. This model, thus, allows to assess, first, the effects of such replacement on energetic metabolism, ischemia-reperfusion injury and cardioprotection, and second to investigate whether these roles are related to specific properties of Cx43.

Despite the observed reduction in body and cardiac weights in HOM Cx43KI32 mice, previous studies have demonstrated that substitution of Cx43 by Cx32 did not induce major changes in baseline electrical properties or contractile function, as assessed by echocardiographic studies and ECG measurements (Plum et al., 2000; Rodriguez-Sinovas et al., 2010). Furthermore, our present data demonstrate lack of differences in PP and left ventricular function, as well as in myocardial electrical impedance, conduction velocity and action potential parameters under normoxic conditions in isolated mice hearts from these animals. However, replacement of Cx43 by Cx32 was associated with altered myocardial energetic metabolism, as demonstrated by a reduction in ATP and an increase in lactate contents. These changes were paradoxically associated with an increased tolerance to ischemia-reperfusion in isolated hearts, and with an inability of myocardium to be preconditioned by ischemia or Dzx. These results, together with data obtained by our group in isolated cardiomyocytes from these animals (Rodriguez-Sinovas et al., 2010), demonstrate that Cx43 has important functions in myocardium that are specific for this connexin isoform, independent of impulse propagation, and that modulate myocardial tolerance to ischemia-reperfusion injury and preconditioning protection.

1.1- Myocardial electrical properties and left ventricular function

Previous studies from our group (Rodriguez-Sinovas et al., 2010) confirmed, using the scrape-and-load technique, the expected lower permeability of GJ in hearts from HOM Cx43KI32 mice, as compared with hearts from WT animals. Gap junctional channels composed by Cx32 have been described to have reduced permeability to several intracellular messengers, as AMP, ADP or ATP, but not to IP3 or adenosine (Niessen et al., 2000; Goldberg et al., 2002).

Despite the lower unitary conductance of Cx32-formed gap junctional channels, as compared with those composed by Cx43 (Goldberg et al., 2002; Harris, 2002), electrophysiological measurements showed only minor differences between groups under baseline conditions. Baseline myocardial electrical impedance, conduction velocity measurements and action potential characteristics, analyzed in isolated mice hearts, were not modified in mutant HOM Cx43KI32 animals. These results indicate that Cx32 is able to replace to a large extent the function of Cx43 regarding propagation of electrical impulse, at least under normoxic conditions. This can be explained, at least in part, by the large safety factor of gap junctional conductance. In fact, it has been previously shown that a marked reduction in Cx43 levels is needed to cause noticeable effects in conduction or in electrocardiographic measurements (Morley et al., 1999; Plum et al., 2000; Eckardt et al., 2006). Importantly, left ventricular function, as assessed by fractional shortening in echocardiographic studies, was not affected by replacement of Cx43 by Cx32 (Rodriguez-Sinovas et al., 2010).

1.2- Effects of replacement of Cx43 by Cx32 on energetic metabolism

Analysis of ^{1}H-NMR spectra showed that replacement of Cx43 by Cx32 modified energetic metabolism in hearts from HOM Cx43KI32 mice. Cardiac extracts obtained from these animals had lower ATP and higher lactate concentrations, as compared with hearts from WT mice. Moreover, ^{31}P-NMR spectroscopy of Langendorff-perfused hearts also confirmed a reduction of PCr /ATP ratio in these mutant mice. Differences in cardiac ATP content between HOM and WT mice could be explained by either an impaired ATP synthesis or an increased ATP consumption. However, this last possibility seems unlikely, as no differences in temperature or heart rate were observed between both genotypes.

The lower ATP and higher lactate concentrations seen in HOM Cx43KI32 mice could be explained by a reduction in the tricarboxylic acid (TCA) cycle turnover. As a consequence of this reduction, less ATP would be produced, and pyruvate would accumulate in the cytoplasm. Pyruvate would, then, be transformed into lactate, leading to its increase inside the cell. It has been reported that TCA cycle flux can be reduced under different situations leading to impaired NAD^+ regeneration, such as ischemic conditions (Schroeder et al., 2009), dysfunction of malate-aspartate shuttle (Lofgren et al., 2010), or impaired mitochondrial complex I activity (Nishiki et al., 1979), among others. Interestingly, mitochondria from mice deficient in Cx43 have been described to have lower respiratory control rate under complex I substrates, as compared with those obtained from WT animals (Boengler et al., 2006; Boengler et al., 2011). Moreover, experiments from our group have observed a similar alteration in complex I-driven respiration in mitochondria from HOM Cx43KI32 hearts (unpublished observations). Therefore, substitution of Cx43 by Cx32 may induce a reduction in TCA cycle turnover by impairing mitochondrial complex I activity. This would lead to a decrease in the synthesis of ATP. These data would support an important role of mitochondrial Cx43 in cardiomyocyte energetic metabolism, and that this function cannot be assumed by Cx32.

1.3- Effects of replacement of Cx43 by Cx32 on myocardial tolerance to ischemia-reperfusion

Previous studies have suggested a role for GJ intercellular communication in spreading of cell death during myocardial reperfusion (Garcia-Dorado et al., 1997; Rodriguez-Sinovas et al., 2004), and also in other tissues and situations (Lin et al., 1998; Rami et al., 2001). However, the lack of specificity of available GJ uncouplers used in previous works made necessary to develop new strategies to study the role of Cx43 in cell death during myocardial infarction, including the use of transgenic mice models. In our present approach, results obtained in isolated hearts from HOM Cx43KI32 mice showed that replacement of Cx43 by Cx32 increased tolerance to myocardial ischemia-reperfusion injury. Cx32 is a connexin isoform with reduced permeability to K^+ and some intracellular messengers (Niessen et al., 2000; Goldberg et al., 2002). Thus, the increased resistance to ischemia-reperfusion injury observed in our

model could be explained by a reduction in GJ-mediated spreading of injury. However, experiments performed in isolated cardiomyocytes from HOM Cx43KI32 mice have also showed reduced LDH release after simulated ischemia-reperfusion (Rodriguez-Sinovas et al., 2010). This result would indicate that the protective effect against ischemia-reperfusion seen after replacement of Cx43 by Cx32 was, at least in part, independent of GJ intercellular communication.

This increased tolerance to ischemia-reperfusion injury may appear surprising in face of the reduced ATP content observed in hearts from HOM Cx43KI32 mice under normoxic, baseline conditions. However, previous studies have shown that reduced TCA cycle and ATP synthesis by inhibition of mitochondrial complex I with amobarbital (Stewart et al., 2009), or impaired malate-aspartate shuttle (Lofgren et al., 2010) are associated with increased resistance to ischemia-reperfusion. Furthermore, deletion of thioredoxin-interacting protein (Txnip), which has been suggested to redirect glycolytically derived pyruvate away from mitochondria towards cytosolic lactate production, thus enhancing anaerobic glycolysis and reducing mitochondrial ATP production rate, has been also associated with an increased resistance to ischemia-reperfusion injury in isolated mice hearts (Yoshioka et al., 2012).

Although the increased tolerance to ischemia-reperfusion injury in HOM Cx43KI32 mice could be explained by the metabolic changes observed in NMR studies, we cannot discard that other mechanisms could have contributed, at least in part, to this effect. Cx43 hemichannels have been described to open transiently during ischemia, contributing to Ca^{2+} overload and cell swelling in cardiomyocytes submitted to simulated ischemia-reperfusion (Shintani-Ishida et al., 2007). Differences in hemichannel permeability and distribution of both connexin isoforms might also have contributed to the endogenous protection observed in the HOM genotype. However, no data on the distribution or function of Cx32 hemichannels in HOM mice hearts is available. In this regard, our immunohistological analysis suggested a similar distribution pattern of both connexin isoforms in hearts from HOM and WT animals. Both are mainly located within the cell poles forming GJ plaques.

Unfortunately, our NMR studies do not allow to accurately determine ATP levels during ischemia in mice hearts, due to a low signal-to-noise ratio. In our ischemia-reperfusion experiments, rigor onset occurred at about 3-4 minutes of ischemia, with no differences between groups. Ischemic rigor contracture develops when cytosolic ATP is reduced to a critical concentration (Piper et al., 2003), and thus,

the results indicate that, despite the lower baseline ATP concentration in HOM Cx43KI32 mice, the time course of ATP depletion during ischemia was similar in all groups. However, despite this similar time course of rigor contracture in both groups, the maximal intensity of contracture was weaker in HOM Cx43KI32 mice. A possible explanation for this difference is the lower permeability of Cx32-gap junctional channels, as GJ have been described to spread ischemic rigor between paired cardiomyocytes (Ruiz-Meana et al., 2001).

1.4- Effects of replacement of Cx43 by Cx32 on cardiac susceptibility to preconditioning protection

Our results show that susceptibility to IPC and Dzx protection is abolished when Cx43 is completely replaced by Cx32, and that 50% substitution of Cx43 expression in HET mice is enough to abolish pharmacological preconditioning by Dzx, but not protection by IPC. Although it could be argued that failure of preconditioning in HOM Cx43KI32 mice is artifactual and only reflects that hearts from these animals are already maximally protected in the absence of preconditioning, our results with cariporide indicate that this is not the case, as hearts from these mice could be effectively protected by this drug. Moreover, lack of protection with Dzx in HET mice cannot be explained by an endogenous baseline protection, as infarct size in this group of animals, under control conditions, was similar to that observed in WT mice.

Previous studies in $Cx43^{+/-}$ mice suggested a role of Cx43 in IPC protection, as these animals, expressing half of the normal Cx43 content, were unable to be protected by IPC, applied before regional ischemia-reperfusion *in vivo* (Schwanke et al., 2002). Although this role of Cx43 in IPC protection has been proposed to be due to a reduced chemical GJ communication (Miura et al., 2010a), it appears, in fact, to be independent, at least in part, of GJ-mediated cell-to-cell communication (Padilla et al., 2003). Thus, isolated cardiomyocytes deficient for Cx43, lacking functional GJ after the isolation procedure, cannot be protected by ischemic or pharmacological preconditioning (Li et al., 2004; Heinzel et al., 2005). Moreover, the failure of Dzx protection in cardiomyocytes from $Cx43^{+/-}$ mice was associated with a reduction in ROS generation after exposure to the drug (Heinzel et al., 2005), suggesting that the role of Cx43 in preconditioning was related to its involvement in ROS signaling.

Mitochondria play a critical role in the ROS signaling of preconditioning protection (Murphy and Steenbergen, 2008). The presence of Cx43 at the inner mitochondrial membrane has been recently described in mice, rat, pig and human cardiac mitochondria (Boengler et al., 2005; Rodriguez-Sinovas et al., 2006a), where it is oriented with the carboxy-terminal domain at the intermembrane space (Miro-Casas et al., 2009). Inhibition of Cx43 translocation to this localization by geldanamycin, without altering total Cx43 content, abolishes Dzx, but not IPC, protection (Rodriguez-Sinovas et al., 2006a). In addition, the absence of Cx43 results in slowed K^+ influx to the mitochondrial matrix in permeabilized cardiomyocytes from HOM Cx43KI32 mice exposed to K^+ pulses, both in the absence and in the presence of Dzx (Miro-Casas et al., 2009). This effect has been also observed in astrocytes, where lack of Cx43 impaired mitochondrial K^+ uptake (Kozoriz et al., 2010). Interestingly, mitochondrial Cx43 has been recently confirmed in Sca-1^+ stem cells, where it may participate in antiapoptotic signaling (Lu et al., 2009), and its overexpression simulated the cytoprotective effects of preconditioning in stem cells (Lu et al., 2010). The inability of HOM Cx43KI32 hearts to be protected by ischemic or pharmacological preconditioning observed in the present study could be, thus, explained by differences in mitochondrial function induced by replacement of Cx43 by Cx32.

The results obtained in HET animals are also remarkable. Hearts from these animals could not be protected with Dzx, although they were still protected by IPC, suggesting that actions of Dzx are particularly dependent on Cx43. This is consistent with the effects of inhibiting Cx43 translocation to mitochondria with geldanamycin, which resulted in a moderate reduction in mitochondrial Cx43 content and abolished Dzx-induced, but not ischemic, preconditioning (Rodriguez-Sinovas et al., 2006a). The different importance of Cx43 on these two forms of protection could reflect the existence of parallel signal transduction pathways in preconditioning protection. In fact, mitochondrial ROS generation appears to be particularly important in Dzx-induced preconditioning (Andrukhiv et al., 2006). Inhibition of complex I respiration has been shown to reduce ROS generation (Stewart et al., 2009), and it is, thus, tempting to speculate that reduced complex I activity could be the cause of both increased tolerance to ischemia and failure of preconditioning.

1.5- Conclusions

This part of the thesis demonstrates a previously unrecognized role of Cx43 in myocardial energetic metabolism and tolerance to ischemia-reperfusion, and indicates that the important role of Cx43 in cardioprotection is specifically dependent on biophysical-biochemical properties of this connexin isoform

2. Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

A marked reduction in Cx43 expression was achieved in the conditional $Cx43^{Cre\text{-}ER(T)/fl}$ knock-out mice model after treatment with 4-OHT. This model mimics a situation of a decrease in the number of available connexin channels, and allows to determine whether the presence of Cx32 contributed to our previous findings obtained in Cx43KI32 mice (altered myocardial energetic metabolism, increased tolerance to ischemia-reperfusion injury, and modified susceptibility to preconditioning protection). Furthermore, we have used this model to assess a possible involvement of cytosolic protective pathways, including the RISK and SAFE signaling cascades, in these effects. In the conditional $Cx43^{Cre\text{-}ER(T)/fl}$ knock-out model, Cx43 levels are markedly reduced to less than 5% 14 days after the beginning of 4-OHT treatment in $Cx43^{Cre/fl}$ mice (Eckardt et al., 2004). This short time of induction avoids most of the compensatory mechanisms that would appear in other transgenic models. Both $Cx43^{Cre/fl}$ mice, and their genetic controls, $Cx43^{fl/fl}$, were treated either with vehicle or 4-OHT, to assess, in the later, possible interferences of 4-OHT treatment per-se.

Whereas hearts from $Cx43^{fl/fl}$ mice express a normal Cx43 content, either after treatment with vehicle (oil) or 4-OHT, control hearts from $Cx43^{Cre/fl}$ animals, treated with oil, express about half of the normal Cx43 content. This mid genetic deficiency did not induce major changes in body and cardiac weights, or in cardiac contractile function. However, the strong depletion of Cx43 induced in $Cx43^{Cre/fl}$ mice after treatment with 4-OHT was associated with an increased LVdevP. This effect can be related to the prolonged action potential duration that we have observed in this group in our present study (see results on pages 136-137).

2.1- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre\text{-}ER(T)/fl}$ mice on energetic metabolism

Contrary to what happened in cardiac extracts obtained from HOM Cx43KI32 mice, ^{1}H-NMR spectroscopy did not show any significant difference in intracellular concentrations of analyzed metabolites in $Cx43^{Cre\text{-}ER(T)/fl}$ animals, including ATP and lactate, between the four experimental groups. In contrast, ^{31}P-NMR spectroscopy of

isolated, Langendorff-perfused, hearts revealed a decreased PCr /ATP ratio in animals with a marked reduction in Cx43 expression, as happened in isolated hearts from HOM Cx43KI32 mice.

Although we do not have an explanation for the lack of differences in ATP and lactate intracellular concentrations in cardiac extracts, our results obtained in isolated mice hearts confirm the previously described alteration in energetic metabolism in hearts from animals lacking Cx43. Furthermore, results from our western blot analysis confirmed that depletion of Cx43 after 4-OHT treatment in $Cx43^{Cre/fl}$ mice is not restricted to total Cx43 content, but also occurs in mitochondrial fractions. This finding, together with the reduced respiratory control rate under complex I substrates observed in isolated cardiac mitochondria from these mice (Boengler et al., 2006; Boengler et al., 2011), supports, again, an essential role for this protein in mitochondrial function. An alteration in mitochondrial complex I activity would lead to a reduction in TCA turnover, and altered PCr /ATP ratio, as suggested in Cx43KI32 animals.

2.2- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre-ER(T)/fl}$ mice on myocardial tolerance to ischemia-reperfusion

Similar to replacement of Cx43 by Cx32 in HOM Cx43KI32 mice, isolated hearts from $Cx43^{Cre/fl}$ animals treated with 4-OHT, showing very low levels of Cx43 expression, depicted an increased tolerance to ischemia-reperfusion injury, as compared with remaining groups. This protection was even higher than that observed in hearts from HOM Cx43KI32 mice. However, and in contrast to what was seen in Cx43KI32 mice (Rodriguez-Sinovas et al., 2010), cardiomyocytes isolated from $Cx43^{Cre/fl}$ animals treated with 4-OHT showed a similar degree of cell death, as determined by Trypan blue staining after simulated ischemia-reperfusion, as compared with all remaining groups. As cardiomyocytes are not able to form functional GJ after the isolation procedure, these data would support the hypothesis that the increased resistance to ischemia-reperfusion injury observed in our model is dependent mainly on a reduction in GJ-mediated spreading of cell death. In contrast, in our previous model (i.e., the Cx43KI32 mice) we observed that protection involved also GJ-independent mechanisms.

All together, our findings are in agreement with those previously obtained using poorly selective GJ uncouplers (Garcia-Dorado et al., 1997; Rodriguez-Sinovas et al., 2004), and would support the hypothesis that GJ mediate spreading of cell death and hypercontracture during reperfusion (Garcia-Dorado et al., 2004). In the same line are studies showing that $Cx43^{+/-}$mice, having about half of normal Cx43 content, submitted to coronary occlusion without reperfusion, have reduced infarct size (Kanno et al., 2003), and that a rise in gap junctional communication, either by preventing channel closure in mouse hearts (Maass et al., 2009), or by increasing the number of channels by overexpression of Cx32 in mice having normal Cx43 content (Prestia et al., 2011), increased infarction. Although the exact mechanisms have not been completely elucidated, it has been suggested that propagation of injury is mediated by passage of Na^+ from injured to healthy cells, and subsequent exchange by Ca^{2+} through the reverse mode of the NCE (Ruiz-Meana et al., 1999). However, other reports have shown conflicting results. Thus, treatment with the antiarrhythmic peptides rotigaptide and Gap-134, which improved gap junctional communication and reduced the incidence of ventricular arrhythmias, decreased infarct size after regional ischemia-reperfusion in anaesthetized rats (Haugan et al., 2006) and dogs (Hennan et al., 2006; Hennan et al., 2009). Furthermore, a reduction in infarction in $Cx43^{+/-}$ mice could not be confirmed after coronary occlusion followed by reperfusion (Schwanke et al., 2002). Differences between our data and these last studies are not clear, but it has been suggested that depending on the severity of injury, GJ may have positive effects, spreading survival agents or diluting cytosolic changes induced by different manoeuvres (Yasui et al., 2000; Garcia-Dorado et al., 2004).

Although our data suggest that a reduction in GJ communication may explain the increased tolerance to ischemia-reperfusion injury in $Cx43^{Cre/fl}$ animals treated with 4-OHT, we cannot completely exclude that other mechanisms are still playing a minor role. Our ^{31}P-NMR spectroscopy data, performed in isolated, Langendorff-perfused, hearts, have suggested an impaired energetic metabolism, with a reduced PCr /ATP ratio. As commented before, previous studies have associated a reduction in TCA cycle turnover and ATP synthesis, as after inhibition of mitochondrial complex I with amobarbital (Stewart et al., 2009), or impaired malate-aspartate shuttle (Lofgren et al., 2010), with an increased resistance to ischemia-reperfusion. Furthermore, deletion of Txnip, which has been suggested to redirect glycolytically derived pyruvate away from mitochondria towards cytosolic lactate production, thus enhancing anaerobic glycolysis

and reducing mitochondrial ATP production rate, has been also associated with an increased resistance to ischemia-reperfusion injury in isolated mice hearts (Yoshioka et al., 2012).

A baseline activation of protective cytosolic cascades, including the RISK and SAFE pathways, could, hypothetically, have contributed to protection against infarction in Cx43-deficient animals. Both cascades include a group of survival protein kinases as, among others, PI3K, Akt, ERK-1/2, endothelial nitric oxide synthase (eNOS) and GSK3β, for the RISK pathway, and JAK and STAT3 for the SAFE pathway. Both pathways have been suggested to be involved in protection by both ischemic pre- and postconditioning (Cohen and Downey, 2011; Hausenloy et al., 2011), converging at the mitochondria where they may modulate function of different structures, including opening of mitoK_{ATP} channels or the MPTP. Protective maneuvers activate these cascades by inducing sequential phosphorylation of these kinases (Tong et al., 2002; Krieg et al., 2004; Hausenloy et al., 2005). The only exception is GSK3β, that is phosphorylated and inactivated during pre- and postconditioning, an effect that has been associated with protection (Tong et al., 2002; Juhaszova et al., 2004). To exclude the possibility of a baseline activation of these cascades in $Cx43^{Cre/fl}$ animals treated with 4-OHT, we tested the phosphorylation (i.e. activation) state of Akt, ERK-1/2, GSK3β, and STAT3, together with total expression of the proteins, in cardiac extracts obtained from normoxic isolated mice hearts. Our results showed a significant reduction in the phosphorylation state of GSK3β in Cx43-deficient mice, and a similar trend for ERK-1/2. These results argue, thus, against a baseline activation of the RISK pathway in $Cx43^{Cre/fl}$ animals treated with 4-OHT. Regarding the SAFE pathway, phosphorylation of STAT3 was almost absent in hearts from all groups of animals, with no differences between them, thus excluding also a baseline activation of this cytosolic signaling cascade.

2.3- Effects of a marked reduction in Cx43 expression in $Cx43^{Cre-ER(T)/fl}$ mice on cardiac susceptibility to preconditioning protection

Protection by IPC was determined in isolated mice hearts submitted to 4 cycles of transient ischemia-reperfusion before 40 min of global index ischemia followed by

flow restoration. As seen in the results section, infarct size was significantly reduced by IPC, as compared with controls, in hearts from $Cx43^{fl/fl}$ mice, either treated with vehicle or 4-OHT, which express a normal Cx43 content, and from $Cx43^{Cre/fl}$ animals treated with oil, having about half of the normal Cx43 content. In contrast, injury was not reduced in hearts from $Cx43^{Cre/fl}$ animals treated with 4-OHT, having very low levels of myocardial Cx43. However, assessment of protection in this group has the limitation of the very low infarct size shown by hearts from these animals under control conditions.

In our previous study in Cx43KI32 mice, we observed that replacement of Cx43 by Cx32 abolished protection by preconditioning, especially that of Dzx. This happened not only in HOM Cx43KI32 animals, but also in HET mice, having about 50% of normal Cx43 content. For this reason, and due to the low infarctions seen in hearts from $Cx43^{Cre/fl}$ animals treated with 4-OHT, we decided to test the effects of pharmacological preconditioning with Dzx in $Cx43^{Cre/fl}$ animals treated with vehicle. As expected, hearts from $Cx43^{fl/fl}$ animals, expressing a normal Cx43 content, were protected by both ischemic and pharmacological preconditioning. In contrast, hearts from $Cx43^{Cre/fl}$ mice treated with vehicle, expressing about 50% of the protein, could not be protected by pre-treatment with Dzx.

As commented in section 1.4 of discussion, previous studies have proposed an essential role of Cx43 in preconditioning protection, as isolated cardiomyocytes from $Cx43^{+/-}$ animals were unable to be protected by IPC (Li et al., 2004) and Dzx, whose failure was associated with attenuated ROS generation (Heinzel et al., 2005). These findings suggested that ROS signaling is involved in the role of Cx43 in preconditioning protection. Mitochondria play a critical role in ROS signaling during preconditioning (Murphy and Steenbergen, 2008). In this sense, the presence of Cx43 in the mitochondria (Boengler et al., 2005) seem to be especially relevant in the case of protection with Dzx, a drug supposed to cause protection by increasing ROS production through actions on mitoK_{ATP} channels (Rodriguez-Sinovas et al., 2006a). Our results with Dzx, and those obtained with hearts from Cx43KI32 mice, thus, support an essential role of Cx43 in mitochondrial ROS signaling. The importance of mitochondrial Cx43 in protection has been also highlighted by a recent study suggesting that it may counteract the Ca^{2+}-induced MPTP opening in rat brain mitochondria, but not in rat liver mitochondria, lacking Cx43 (Azarashvili et al., 2011).

The absence of Cx43 at the mitochondria may have important functions in the physiology of the organelle. Replacement of Cx43 by Cx32 resulted in slowed K^+ influx

to mitochondrial matrix in permeabilized cardiomyocytes (Miro-Casas et al., 2009). Furthermore, a reduced respiratory control rate under complex I substrates has been observed in isolated cardiac mitochondria from Cx43-deficient mice (Boengler et al., 2006; Boengler et al., 2011). These effects, which are probably the cause of the altered energetic metabolism observed in our NMR studies, could be also responsible for the lack of protection observed in hearts from these animals. Inhibition of complex I respiration is also known to reduce ROS generation (Stewart et al., 2009). It is, thus, tempting to speculate that either a reduced complex I activity or mitoK_{ATP} channel function could be the cause of the failure of preconditioning.

However, it is still possible that lack of protection by IPC in Cx43-deficient mice was in fact due to lack of activation, in these animals, of cytosolic signaling cascades, including the RISK and SAFE pathways. To exclude this possibility we tested, in extracts obtained from isolated mice hearts submitted to 4 cycles of transient ischemia-reperfusion, whether there is a differential action of these pathways in Cx43-deficient animals. Our results demonstrate an increased phosphorylation of GSK3β and STAT3, and a similar trend for ERK-1/2, in all hearts submitted to IPC, independently of group allocation, suggesting that both RISK and SAFE pathways are equally activated independently of Cx43 expression. These findings would support that activation of both pathways is upstream to the effects of Cx43 deficiency. In contrast, other authors have shown that a reduction in Cx43 content to 20% of normal level by Cx43-siRNA in H9c2 cardiomyocytes, which was shown to abolish protection by pre-treatment with δ-opioid receptor agonists and endothelin-1, was associated with loss of phosphorylation of Akt and GSK3β, but not with attenuated ROS production (Ishikawa et al., 2012). This last study demonstrated co-immunoprecipitation of Cx43 with the β subunit of G protein, which made authors to suggest that Cx43 (probably sarcolemmal Cx43) contributes to activation of PI3K-Akt-GSK3β signaling pathway as a co-factor of Gβ in H9c2 cardiomyocytes (Ishikawa et al., 2012). Lack of agreement between these last results and our data could be explained by differences in the functions of the two Cx43 populations (sarcolemmal vs. mitochondrial), in the models used, and/or in the protective manoeuvre applied.

2.4- Conclusions

This part of the thesis demonstrates that the role of Cx43 in myocardial energetic metabolism, tolerance to ischemia-reperfusion injury, and susceptibility to preconditioning protection, previously described in Cx43KI32 mice, was not dependent on the presence of Cx32. Moreover, the effects of Cx43-deficiency on the reduction of cell death after ischemia-reperfusion and on the lack of protection by preconditioning are not due to a different activation of RISK and SAFE cytosolic signaling pathways.

3. Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on the incidence of ventricular arrhythmias, and on passive and active myocardial electrophysiological properties, during ischemia-reperfusion

The aim of this part of the thesis was to analyze the effects of both an extreme reduction in the number of GJ channels or in their unitary conductance, on the incidence of ventricular arrhythmias during myocardial ischemia-reperfusion. To reduce the number of available GJ channels, we used $Cx43^{Cre\text{-}ER(T)/fl}$ mice, in which a marked decrease in Cx43 expression is achieved, as described in previous sections, by 4-OHT administration. To specifically reduce unitary GJ conductance, we used the Cx43KI32 mouse model, in which the coding region of Cx43 is replaced by that of Cx32.

Our results demonstrate that a reduction in the number of available GJ channels or in their permeability/conductance, two strategies that have been associated with reduced infarct size after myocardial ischemia-reperfusion in previous sections of this thesis, increases susceptibility to spontaneous and inducible ventricular arrhythmias under normoxic conditions, during ischemia and during reperfusion. This increased arrhythmogenesis occurs despite the absence, in HOM Cx43KI32 mice, of major changes in cardiac baseline macroscopic electrical properties, including conduction velocity and myocardial electrical impedance, and is similar to that seen in $Cx43^{Cre/fl}$ mice treated with 4-OHT. These results must be taken into account when considering translation of treatments aimed to reduce Cx43-mediated intercellular communication to patients undergoing myocardial infarction.

3.1- Effects of a marked reduction in the number of available Cx43 gap junctional channels

Electrophysiological studies have demonstrated that GJ channels composed of Cx43 can reside in at least three different states: closed, opened and residual (Bukauskas and Peracchia, 1997). Most of pharmacological agents used to uncouple GJ, as heptanol, halogenated anaesthetics, glycyrrhetinic acid and derivates, keep Cx43 channels in the closed state, reducing open channel probability (Takens-Kwak et al., 1992; He and Burt, 2000). However, the use of GJ uncouplers to assess the role of Cx43-formed GJ in arrhythmogenesis has important limitations, as they have effects on

other non-junctional ionic currents. Instead, genetic Cx43 deficiency can be considered to mimic treatments reducing the probability of the open state.

Different models of Cx43 deficiency have been used to analyze the role of Cx43 in the susceptibility to ventricular arrhythmias, including $Cx43^{+/-}$ mice (Lerner et al., 2000), animals with progressive cardiac-restricted inactivation of Cx43 (Gutstein et al., 2001a; Danik et al., 2004), and the $Cx43^{Cre\text{-}ER(T)/fl}$ mice model (van Rijen et al., 2004). The general conclusion that can be obtained from these studies is that only when Cx43 content is markedly reduced, the incidence of both spontaneous and inducible arrhythmias is increased (van Rijen et al., 2004; Danik et al., 2004). At these low levels, Cx43 expression has been shown to become heterogeneous, increasing anisotropy (Gutstein et al., 2001a; van Rijen et al., 2004). In contrast, studies performed in models with higher Cx43 content, as $Cx43^{+/-}$ mice, reported inconclusive results, in part due to differences in the experimental settings (Lerner et al., 2000; Betsuyaku et al., 2004; Stein et al., 2009).

Several authors have suggested a relationship between the onset of cell-to-cell electrical uncoupling and ischemic phase Ib arrhythmias (Verkerk et al., 2001; Jie et al., 2008). However, previous studies using Cx43-deficient mice have been performed mostly under baseline or normoxic conditions, and information of the role of Cx43-formed GJ on arrhythmogenesis during ischemia-reperfusion is scant. Our present data demonstrate that the severe reduction in myocardial Cx43 content observed in $Cx43^{Cre/fl}$ mice treated with 4-OHT is associated with a marked increase in the incidence of ventricular arrhythmias under normoxic conditions, but also during ischemia and during reperfusion. These findings are in agreement with the notion that altered Cx43-mediated intercellular communication plays an important role in ischemia-reperfusion arrhythmias, and limits the possibility of translation of therapeutic strategies aimed to block cell-to-cell communication to reduce ischemia-reperfusion injury, to the clinical scenario. However, we cannot exclude that part of the increased arrhythmogenesis found in these animals lacking Cx43 could be due to other ionic alterations, as action potentials recorded from these mice hearts showed increased dV/dt_{max} and prolonged action potential durations. Although the increased action potential duration is not associated with a similar prolongation in effective refractory periods, the latter being always longer than the former, such increase in action potential duration may be, *per se*, arrhythmogenic. On the other hand, the increased dV/dt_{max} in $Cx43^{Cre/fl}$ mice treated with 4-OHT might reflect a compensatory mechanism to sustain conduction in these animals, having a

marked reduction in conduction velocity at baseline. This interpretation is supported by the absence of changes in action potentials in HOM Cx43KI32 mice, having normal conduction velocity at baseline.

Our results demonstrate that Cx43 depletion by 4-OHT treatment causes a significant reduction in conduction velocity under baseline conditions. However, this effect was not observed in hearts from $Cx43^{Cre/fl}$ mice treated with vehicle, having about 50% of normal Cx43 content. These findings are in agreement with previous studies showing that a marked reduction in Cx43 levels is needed to induce changes in conduction velocity (Morley et al., 1999; Jongsma and Wilders, 2000). Despite these differences, the time course of conduction impairment during ischemia was similar in all groups, denoting a similar regulation during this condition.

Contrary to conduction velocity measurements, our study shows that a reduction in Cx43 expression in $Cx43^{Cre/fl}$ mice treated with 4-OHT does not modify baseline myocardial electrical impedance. Measurement of myocardial electrical impedance in intact myocardium, which does not allow to discriminate between intracellular and extracellular resistances, has been proven to be feasible in both isolated and *in situ* cardiac preparations using four-electrode probes (Beardslee et al., 2000; Coronel et al., 2002; Rodriguez-Sinovas et al., 2004). Such methodology was validated in the rabbit papillary muscle model by a modification of the classic linear cable analysis (Kleber et al., 1987). However, application of this methodology in intact hearts has limitations, as cardiac geometry does not fit well with a linear cable. This makes absolute baseline values senseless, as they depend, among others, on the amount of tissue under measurement and on the presence of an extracellular shunt resistance, made up by the perfused fluid. But, on the other hand, relative changes following a particular maneuver can be considered, after normalization against the initial value. Our data show that the maximal changes occurring during ischemia in both myocardial resistivity and phase angle, are significantly attenuated in hearts from $Cx43^{Cre/fl}$ mice, especially after 4-OHT treatment. Due to the lack of an accurate initial resistivity value, we should assume that the maximal change occurring during ischemia represents the maximal degree of uncoupling achieved in each genotype. Our data would suggest, then, an altered cell-to-cell electrical coupling in these mice under normoxic, baseline conditions.

3.2- Effects of a reduction in unitary gap junction conductance

Permeability of Cx43-formed GJ has been proposed to be reduced to a residual state when a gating element of the molecule moves into the vestibule of the channel, narrowing the size of the pore (Bukauskas et al., 2002). However, no drugs able to induce the residual state are currently available. To analyze if a specific reduction in unitary gap junctional conductance might constitute a promising therapeutic strategy to reduce infarct size without the associated increase in arrhythmogenesis, we used the Cx43KI32 mice model. As it has been mentioned before, Cx32 is a connexin isoform with lower unitary conductance and altered permeability, compared with Cx43 (Goldberg et al., 2002; Harris, 2002). Furthermore, this model allowed us to assess whether the incidence of ventricular arrhythmias, and changes on macroscopic electrical properties during ischemia and during reperfusion, are influenced by the specific regulation of each connexin isoform. In the first part of this thesis, it has been described that these animals have an increased tolerance to myocardial ischemia-reperfusion injury, despite the fact that they did not show major changes in myocardial electrical properties under normoxic conditions.

Our data demonstrate that, similar to a marked reduction in the number of available Cx43-gap junctional channels, a specific decrease in unitary GJ conductance by replacement of Cx43 by Cx32 causes an increased incidence of ventricular arrhythmias under normoxic conditions, during ischemia and during reperfusion, highlighting the importance of Cx43 in maintaining a safe conduction.

In this animal model, replacement of Cx43 by Cx32 did not modify baseline conduction velocity in isolated mice hearts, contrary to what happened in hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT. However, the impairment in conduction observed during global ischemia was significantly more pronounced in hearts from HOM Cx43KI32 animals, as compared with WT mice. Such different time course during ischemia would indicate the existence of differences in regulation between Cx43 and Cx32-formed GJ in our model. In fact, previous studies have demonstrated that Cx32 is less sensitive to acidification-induced gap junctional closure (Morley et al., 1996). However, a reduced sensitivity to acidification would be expected to delay the impairment in conduction, but not to accelerate it. It is still unknown if other changes in regulation are important for the described differences in conduction velocity during ischemia, and probably for arrhythmia susceptibility in this model. Importantly, the loss of the regulatory domain of Cx43, which

concentrates most of the differences in sequence with other connexin isoforms, has been described to increase the susceptibility to ventricular arrhythmias following acute coronary occlusion (Maass et al., 2009). However, we have to acknowledge that the lack of differences under baseline conditions and the different behavior during ischemia in hearts from HOM Cx43KI32 mice might have an alternative explanation, that might be a low sensitivity of our electrogram analysis. It is possible that our electrogram recordings were devoid of enough sensitivity to assess subtle changes in impulse conduction at baseline, or that the path of activation changed during ischemia in these animals. Despite this limitation, our results demonstrate the existence of marked differences in conduction between hearts from HOM Cx43KI32 and $Cx43^{Cre/fl}$ mice treated with 4-OHT, with very low levels of Cx43 expression. On the other hand, changes in myocardial electrical impedance during ischemia were attenuated in HOM Cx43KI32, probably reflecting, as in $Cx43^{Cre/fl}$ mice treated with 4-OHT, an altered cell-to-cell electrical coupling.

It has been previously demonstrated that at least a 90% reduction in Cx43 expression is needed to observe noticeable effects on cardiac conduction (Jongsma and Wilders, 2000). Replacement of Cx43 by Cx32 is expected to induce only about a 60% reduction in unitary GJ conductance (Harris, 2002). Whereas this could explain the lack of differences on baseline conduction velocity in Cx43KI32 mice, it would, hypothetically, argue against a marked increase in the incidence of ventricular arrhythmias during ischemia-reperfusion. However, and although hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT have an enhanced incidence compared with those from HOM Cx43KI32 animals, our results still demonstrate that HOM Cx43KI32 mice have increased ventricular arrhythmogenesis compared with WT animals. An explanation for the higher than expected incidence of ventricular arrhythmias in HOM Cx43KI32 mice, can be the existence of other factors that could favor their appearance. Changes in regulation, as those previously mentioned, that may be responsible for the faster impairment in conduction seen during ischemia in these hearts, could also be involved in the increased arrhythmogenesis. On the other hand, no changes in action potential characteristics have been observed in these animals. Further studies are needed to completely understand the mechanisms responsible for this enhanced ventricular arrhythmogenesis.

Contrary to our findings in HOM Cx43KI32 mice, Anyukhovsky et al. and Prestia et al. have recently demonstrated that overexpression of Cx32 in WT animals, having normal Cx43 content, is associated with an increased intercellular coupling and a

reduced incidence of reperfusion arrhythmias (but not of arrhythmias during ischemia), together with an increase in infarct size (Anyukhovsky et al., 2011; Prestia et al., 2011). Differences between their results and ours are probably due to the distinct models used. Co-expression of both connexin isoforms may increase the amount of functional gap junctional channels or modify their regulation through formation of heteromeric or heterotypic channels, leading to an increase in cell-to-cell coupling. In contrast, complete replacement of Cx43 by the less conductive Cx32 causes a reduction in cell coupling, as assessed by scrape-and-load experiments (Rodriguez-Sinovas et al., 2010), and suggested by our electrical impedance measurements, thus increasing the incidence of ventricular arrhythmias during ischemia-reperfusion, and reducing infarct size.

3.3- Conclusions

In this part of the thesis, we have demonstrated that both a reduction in the number of available Cx43 gap junctional channels or in their unitary conductance cause an increased incidence of ventricular arrhythmias during normoxia and during myocardial ischemia-reperfusion. Furthermore, data obtained in Cx43KI32 mice suggest that modifications in gap junctional communication, not apparent under normal conditions, may be pathophysiologically important during ischemia-reperfusion, and supports an essential role for Cx43 in arrhythmogenesis during these periods. Differences in channel regulation may, in addition to the lower conductance of Cx32-formed GJ channels, explain the enhanced arrhythmogenesis seen in HOM Cx43KI32 mice hearts. These findings reduce the possibility of translation of these therapeutic strategies to the clinical arena in order to limit infarct size, unless they were applied specifically at the area at risk.

Summary of results and conclusions

Summary of results

1.- Effects of replacement of Cx43 by Cx32 in Cx43KI32 mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

1.1- Replacement of Cx43 by Cx32, a condition mimicking a reduction in GJ unitary channel conductance, did not modify baseline cardiac function under normoxic conditions.

1.2- The expression pattern of both connexin isoforms was similar in the three different genotypes, being mainly distributed at the cell poles, within the intercalated disks.

1.3- Replacement of Cx43 by Cx32 reduced ATP and increased lactate content in cardiac extracts from HOM Cx43KI32 mice, as determined by ^{1}H-NMR spectroscopy. In addition, ^{31}P-NMR spectra from Langendorff-perfused hearts showed a reduced PCr /ATP ratio in hearts from HOM Cx43KI32 mice. All together, these results suggest an impaired energetic metabolism in hearts from HOM Cx43KI32 mice.

1.4- Isolated hearts from HOM Cx43KI32 mice showed an increased tolerance to global ischemia-reperfusion injury, assessed by a reduction in LDH release and infarct size after 40 minutes of ischemia and 60 minutes of reperfusion, as compared with WT hearts.

1.5- IPC and pharmacological preconditioning with Dzx reduced LDH release and infarct size in isolated hearts from WT mice submitted to ischemia-reperfusion. In contrast, only IPC protected hearts from HET animals, and both IPC and Dzx failed to afford protection in hearts from HOM Cx43KI32 mice.

2.- Effects of a marked reduction in Cx43 expression in Cx43$^{Cre\text{-}ER(T)/fl}$ mice on energetic metabolism, myocardial tolerance to ischemia-reperfusion and susceptibility to preconditioning protection

2.1- Treatment with 4-OHT in Cx43$^{Cre/fl}$ mice markedly reduced Cx43 content in both total and mitochondrial fractions, and induced a significant increase in baseline LVdevP. Other parameters of cardiac function were not altered.

2.2- The distribution pattern of Cx43, that was almost restricted to the intercalated disks in control Cx43$^{fl/fl}$ hearts, either treated with vehicle or 4-OHT, became

more heterogeneous in cardiac samples from $Cx43^{Cre/fl}$ mice treated with vehicle, and expression was almost absent after treatment with 4-OHT in this genotype.

2.3- A marked reduction in Cx43 content in $Cx43^{Cre/fl}$ mice treated with 4-OHT was not associated with changes in the intracellular concentration of analyzed metabolites, as determined by ^{1}H-NMR spectroscopy. In contrast, ^{31}P-NMR spectra from Langendorff-perfused hearts showed a reduced PCr /ATP ratio in these animals, which is suggestive of an altered energetic metabolism.

2.4- Depletion of Cx43 in isolated hearts from 4-OHT-treated $Cx43^{Cre/fl}$ mice conferred an increased tolerance against global myocardial ischemia-reperfusion injury, as compared with the remaining groups.

2.5- An increased resistance to simulated ischemia-reperfusion injury was not observed in isolated cardiomyocytes from $Cx43^{Cre/fl}$ mice treated with 4-OHT.

2.6- The increased tolerance to ischemia-reperfusion injury observed in isolated hearts from $Cx43^{Cre/fl}$ mice treated with 4-OHT was not associated with a baseline activation of cytosolic protective signaling cascades, including the RISK and SAFE pathways.

2.7- Infarct size and LDH release were significantly reduced by IPC, as compared with controls, in hearts from $Cx43^{fl/fl}$ mice, either treated with vehicle or 4-OHT (expressing a normal Cx43 content), and from $Cx43^{Cre/fl}$ animals treated with oil (having about half of the normal content). In contrast, injury was not reduced in hearts from $Cx43^{Cre/fl}$ animals treated with 4-OHT, having very low levels of myocardial Cx43.

2.8- Pharmacological preconditioning with Dzx was able to induce protection against ischemia-reperfusion injury in hearts from $Cx43^{fl/fl}$ mice, but failed to protect hearts from $Cx43^{Cre/fl}$ mice treated with oil.

2.9- IPC induced an increased phosphorylation of GSK3β and STAT3 in all hearts independently of group allocation, with a similar trend for ERK-1/2, denoting that both RISK and SAFE pathways are equally activated independently of Cx43 expression. These results suggest that activation of both pathways is upstream to the effects of Cx43 deficiency.

3.- Effects of both replacement of Cx43 by Cx32, and a marked reduction in Cx43 expression, on the incidence of ventricular arrhythmias, and on passive and active myocardial electrophysiological properties, during ischemia-reperfusion

3.1- Replacement of Cx43 by Cx32 in Cx43KI32 mice, and a marked reduction in Cx43 content in $Cx43^{Cre/fl}$ mice treated with 4-OHT, increased the incidence of both spontaneous and inducible ventricular tachyarrhythmias in isolated mice hearts under normoxic conditions, and during regional ischemia-reperfusion.

3.2- This arrhythmogenic effect was not associated with major changes in baseline myocardial conduction velocity, tissue electrical impedance or transmembrane action potential characteristics in isolated hearts from HOM Cx43KI32 mice.

3.3- In contrast, the pro-arrhythmic effect induced by a marked reduction in Cx43 expression was associated, under baseline, normoxic, conditions, with a decreased conduction velocity, whereas transmembrane action potential recordings showed an increased dV/dt_{max} and a prolongation in action potential duration.

3.4- The maximal increase in myocardial electrical resistivity observed during ischemia was significantly reduced in hearts from Cx43-deficient animals (both in HOM Cx43KI32 mice and in $Cx43^{Cre/fl}$ mice treated with 4-OHT).

3.5- Impairment in conduction during myocardial ischemia was significantly more pronounced in hearts from HOM Cx43KI32 mice, as compared with WT, probably denoting differences in regulation between both connexin isoforms.

Conclusions

1. Cx43 has an important, and previously unknown, modulatory effect on myocardial energetic metabolism, which is not shared by other connexin isoforms.

2. Cx43 deficiency enhances tolerance to myocardial ischemia-reperfusion injury, at least in part, through GJ-dependent mechanisms.

3. Cx43 plays an essential role in preconditioning protection, especially in that triggered by diazoxide, probably mediated by its mitochondrial localization.

4. The increased tolerance to ischemia-reperfusion injury and the absence of protection by preconditioning conferred by Cx43 deficiency are independent of a differential activation of the cytosolic RISK and SAFE pathways, suggesting that activation of both pathways is upstream to the effects of Cx43 deficiency.

5. A reduction in both GJ unitary channel conductance, and in the number of available GJ channels, is associated with an increased incidence of ventricular tachyarrhythmias during normoxia and ischemia-reperfusion, together with changes in cardiac electrophysiological properties. These findings indicate that the possibility of translation of these therapeutic strategies to the clinical arena requires that they could be applied locally at the area at risk.

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