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CELLULAR MODELS OF HYPOXIA-REOXYGENATION FOR THE STUDY OF NEW MOLECULES WITH THERAPEUTIC POTENTIAL IN ISCHEMIC HEART DISEASE

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Ischemic heart disease

Ischemic heart disease and its most severe manifestation, i.e. myocardial infarction, is a major cause of death and disability worldwide and myocardial infarct size is a major determinant of prognosis (Schmidt et al., 2014).

The pathogenic occurrences in the infarcted heart are dependent on ischemia/reperfusion (I/R).

I/R is a process whereby the initial damage caused to tissue by compromised blood flow and the related metabolic starvation is then compounded by additional and more severe injury caused by re-oxygenation upon blood flow restoration. The compromised blood flow is a result of narrowing of the coronary artery (Liem et al., 2007). It is therefore essential to restore coronary flow to the ischemic myocardium by interventions such as angioplasty, thrombolytic treatment or coronary bypass surgery (Jennings and Reimer, 1991).

Patients, who are able to overcome the acute phase of I/R, face long-term complications due to adverse myocardial remodeling, mainly impaired myocardial contractile function and heart failure (Jaber and Holmes, 2007). In particular, cardiac dysfunction caused by nonlethal myocardial infarction affects an ever-increasing number of subjects, depending on the ageing of population, with a mean 5-year survival of 50-70%. Hence, there is a major interest in the identification of new therapeutic agents that can prevent or reduce myocardial injury.

Ischemia/reperfusion (I/R) pathophysiology

During I/R, there is a primary damage of the coronary endothelium leading to impaired production of nitric oxide ($NO^{\bullet}$) and failure of endothelium-dependent functions, chiefly vasodilatation and prevention of leukocyte and platelet adhesion. This triggers a cascade of events that include recruitment of harmful reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl anion (OH^-). The cellular components susceptible to damage by ROS are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (nitration, oxidation and denaturation), carbohydrates and nucleic acid (Jaeschke and Woolbright, 2012). Furthermore, ROS determine the release of histamine, a powerful arrhythmogenic factor, and the increase of intracellular calcium concentration; these actions produce an irreversible damage, apoptosis and finally necrosis of cardiomyocytes (McCord 1985; Simpson 1988).

The process is accompanied by inflammatory cell recruitment secondary to endothelial injury (Kokura et al., 2002). Indeed, activated inflammatory leukocytes, especially neutrophils and, at a later stage, macrophages, provide a major contribution to oxidative stress through the production of both superoxide anion ($O_2^{\bullet -}$) and $NO^{\bullet}$, which react to generate peroxynitrite ($ONOO^-$), a major oxidant for lipid membranes, sulfhydryl residues, aromatic residues of proteins and other biomolecules (Bencini et al., 2010).

In conclusion, the mechanisms of I/R injury that induce progression of cardiac damage and development of myocardial fibrosis and heart failure are multiple and complex (Eefting 2005): they basically involve oxidative stress (McCord 1985; Simpson 1988) and inflammation (Frangogiannis 2002).

Aims of the study

The study described in this thesis was designed and performed to test the protective action on I/R damage by two molecules, a natural one, the peptide hormone relaxin (RLX) which has been demonstrated to possess prominent cardioprotective actions, and a synthetic one, a Mn^{II} complex with tetraamine dicarboxylic acid 4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetic acid, also termed $Mn^{II}(Me_2DO2A)$, endowed with ROS-scavenging characteristics.

Relaxin

Relaxin (RLX) is a peptidic hormone produced mainly by the corpus luteum, the uterus and the placenta during pregnancy. Its main function is to facilitate childbirth, helping the tissues of the birth canal to relax and the interpubic ligament to elongate. The discovery of the RLX hormone dates back to the second half of the 1920s.

Discovery

RLX was first discovered by Frederick Hisaw (1926) as a substance that could facilitate parturition, based on its observed ability to soften and expand the pubic ligament prior to delivery in pregnant female gophers and guinea pigs and was subsequently named for these actions. Hisaw noticed that the injection of serum from pregnant guinea pigs or rabbits into virgin guinea pigs induced relaxation of the pubic ligament when administrated shortly after estrus (Hisaw 1926). Further studies conducted by Hisaw in 1927, located this “relaxing” factor in the pig corpus luteum and rabbit placenta (Hisaw 1927), and the hormone was formally named “relaxin” after it was extracted from the pig corpus luteum in 1930 (Fevold et al., 1930).

After this initial discovery, research on RLX declined for the next 15 years due to the economic depression in the 1930s and world conflict in 1940s, as well as limitations in peptide isolation and available RLX bioassays (reviewed in Bani 1997).

However, there was a surge of research interest on RLX from the late 1940s through the early 1960s, during which time, a better understanding of the physiological roles of RLX in pregnancy and parturition was achieved.

RLX was reportedly shown to have several effects on the reproductive system, including its ability to promote expansion of the interpubic ligament in estrogen-primed mice (Hall 1947; Hall 1948), mediate relaxation of the uterine myometrium in estrogen-primed guinea pigs (Krantz et al., 1950) and induce cervical softening in estrogen-primed cattle (Graham and Dracy 1953).

These physiological actions of RLX were centered on its ability to regulate collagen metabolism to facilitate pregnancy and successful parturition in females (Sherwood 2004); and thus, RLX was often referred to as a pregnancy-related hormone.

During the late 1950s and early 1960, human studies exploiting the use of RLX as a therapeutic agent were supported by Warner-Chilcott laboratories, which generated an impure preparation of

porcine RLX (Releasin). Releasin was found to have several beneficial effects, being able to prevent premature birth by inhibiting uterine contractility, reduce the duration of child-birth by softening the cervix, and increase skin elasticity in patients with progressive systemic sclerosis (Sherwood 2004). However, clinical studies with Releasin in the mid-1960s were discontinued due to the lack of consistent effectiveness, safety issues and the failure to meet the stringent requirements of the United States Food and Drug administration (FDA) (Evans 1959; Erikson and Unemori 2001).

Over the years, research on RLX has significantly increased due to improved methods for its isolation and purification (Sherwood and O'Byrne 1974), the generation of sensitive and reliable bioassays to measure its level in the blood, and the development of methods to determine its protein structure, amino acid sequence and biological actions which preluded to the production of human RLX by recombinant DNA technology well suited for human use, as better specified in the following chapters (Sherwood 2004; Bathgate et al., 2006; Bathgate 2013).

In the last thirty years it has been demonstrated that RLX, historically classified as a hormone pertaining only to the reproductive sphere, is not a mere hormone of reproduction, but can influence the function of many different organs and tissues (Bani 1997; Dschietzig and Stangl 2002 Sherwood 2004). Thus, RLX can currently be regarded as one of the most pleiotropic hormones ever known.

Genes

Developing recombinant DNA technologies enabled RLX researchers to clone RLX cDNAs from various species, which showed that it is structurally related to insulin. Despite this link, there is only an approximately 25% sequence homology between RLX and insulin (Bathgate et al., 2006), and there are no common cellular effects between the two hormones (Bennett 2009).

It is now well-documented that RLX belongs to a distinct of family of peptide hormones that diverged from insulin and the insulin-like growth factors early in vertebrate evolution (Hsu 2003; Wilkinson et al., 2005; van der Westhuizen et al., 2008). RLX family peptides are distinguished by their ability to bind and activate distinct GPCRs, as opposed to the tyrosine kinase receptors that are activated by peptides of the insulin family (Hsu 2003; Wilkinson et al., 2005; van der Westhuizen et al., 2008).

Screening of genomic libraries and database searches have shown that the RLX peptide family in humans and higher primates are encoded by seven genes, which includes three RLX genes: RLN1,

RLN2, RLN3 and four insulin-like (INSL) peptides genes; INSL3, INSL4, INSL5 and INSL6 (van der Westhuizen et al., 2008; Bathgate 2013). On the other hand, most other species consist of five of these genes which include: RLN1 (equivalent to human RLN2), RLN3, INSL3 INSL5 and INSL6 (equivalent to human RLN3, INSL3, INSL5 and INSL6, respectively) (Bathgate 2003; Samuel 2007).

In humans, the RLN1 and RLN2 genes are located on chromosome 9, in close vicinity to genes INSL4 e INSL6, whereas RLN3 is located on chromosome 19, near INSL3; INSL5 is found on chromosome 1 (Bathgate et al., 2002; Hsu et al., 2003). In mice, instead, RLN1 is located on chromosome 19, in the vicinity of INSL6, while RLN3 maps on chromosome 8, near INSL3.

The products of the human genes RLN1, RLN2 and RLN3 are named respectively H1, H2 and H3 RLX, while the products of murine genes and of rats RLN1 and RLN3 are called relaxin and relaxin 3 (Bathgate et al., 2006).

Although the peptides of these genes share low amino acid sequence homology, phylogenetic analysis revealed that they are evolved from a same ancestral gene, the RLN3 gene (Hsu 2003; Wilkinson et al ., 2005). Furthermore, the genes encoding the human relaxin family peptides were found to share a similar structure and synthesize similar properties (Hsu 2003).

It is now well established that the product of human RLN2 gene (H2 RLX) is the functional orthologue of the RLN1 gene product (relaxin) of non-primate species. H2 RLX and its species equivalent RLX peptide are the major stored and circulating forms of RLX in their respective species (Samuel et al., 2007).

Structure

RLX is a polypeptidic hormone of 6 kDa with a structure similar to insulin, with similar molecular weight, number and length of chains and disulfide bonds (Bathgate et al., 2006).

RLX, like insulin, is formed by two chians, A e B (Figure 1); A chain consists of 24 amino acid residues (25 in mice) with 2 α-helix structures in A3-9 and A13-20 position, whereas the B chain is made up of 29 amino acid residues with only one α-helix structure in B7-22 position. The chains are bound covalently by two disulfide bonds; besides, an intra-chain disulfide bond (in the A chain) stabilizes the tertiary structure (Bathgate et al., 2006). The glycine in B8 e B20 position and half of the cysteines of the disulfide bonds are essential for the correct “insulin-like” folding. It is, thus, clear how RLX and insulin share a similar structure (Bathgate et al., 2006).

In various species, RLX maintains a scarce homology of the amino acid sequence, comprised between 30% and 60%. This is at variance with a highly preserved localization of the disulfide bonds and of the cysteines, which seem to yield a tertiary structure similar for all isoforms (Bryant-Greenwood et al., 1994).

The molecular analysis has led to identify relaxin's amino acid residues preserved during evolution: in the A chain cysteines are constant in A10, A11, A15 position, the glycine in A14, the lysines in A9 e A17 and the arginines in A18 e A22, while in B chain, the glicines in B12 e B14 and the arginines in B13 e B17.

The arginines B13 e B17 are found close to the disulfide bond and carry out a fundamental role for bioactivity, as they are involved in the interaction ligand-receptor (Bullesbach and Schwabe, 2005); even their minimum variations cause the lack of the interaction with receptor.

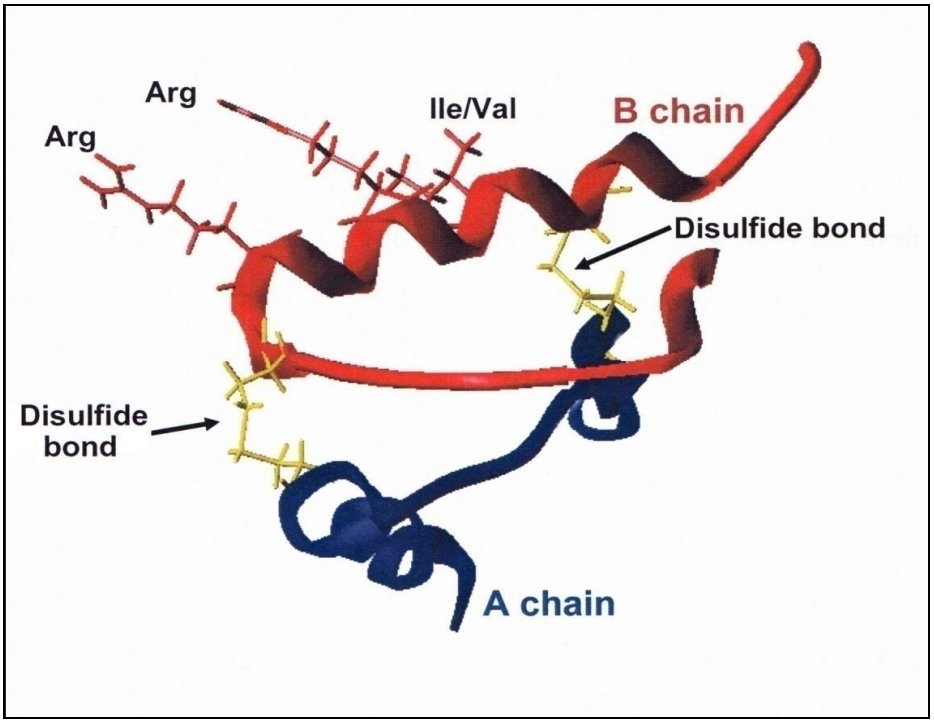

Fig.1 Schematic structure of RLX (Sherwood, *Endocr Rev* 25:205–34, 2004)

Synthesis

RLX, like insulin, is synthesized in the form of a precursor, known as pre-pro-relaxin. The latter has a molecular weight of 23 KDa and is made up of a single chain that includes the signal peptide, B chain, the connection peptide C and A chain.
The maturation process begins by the action of enzymes of the rough endoplasmic reticulum, that carry out a cut of the signal peptide (3KDa) at the carboxyterminal end of B chain; from this cut pro-relaxin is obtained.
The complete maturation occurs presumably in the Golgi apparatus: the PC-1 e PC-2 convertase, involved in the synthesis of insulin by pro-insulin, carry out the cut between C peptide and carboxyterminal end of A chain, while PC-3 determines the cut between C peptide and the B chain (Smeekens et al., 1992). C peptide could be involved in the folding of the protein, as it seems to direct the correct formation of the disulfide bonds between A chain and B chain.
The pre-pro-relaxin is without biological activity, while pro-relaxin, even though endowed with C peptide, has a biological activity compared to the mature molecule (Zarre et al., 2001).

RLX sources

Reproductive tissues

The expression of H1 RLX is limited to the decidua and the placenta, even if the biological role of this isoform still remains unknown. H2 RLX in human and relaxin in mice and rats are produced in high levels during pregnancy by the placenta, the uterus and - above all - by the corpus luteum. The latter is its main source in various animal species (Skott and Carter, 2002; Sherwood, 2004; Bathgate et al., 2002; Samuel et al., 2003). Secondary sources of H2 RLX have been located in the mammary glands and the fallopian tubes (Bryant-Greenwood et al., 1987; Mazoujian and Bryant-Greenwood, 1990), while in mice they are the mammary glands (Peaker et al., 1989) and in rats they are the uterus, the placenta and the mammary glands (Gunnersen et al., 1995). Relaxin 3 in mice is produced by the luteal cells and it is expressed also in the ovary, even if the two forms do not seem to be identical (Bathgate et al., 2002).

Although RLX is often associated as a female pregnancy-related hormone, it is also produced in males but in smaller quantities. The expression of H1 RLX is limited to the prostate and, even if its biological role remains unknown, small quantities are found in the seminal fluid (Gunnersen et al., 1996, Yki-Jarvinen et al., 1983). The expression of H2 RLX and its mRNA has been detected in the prostate and in the seminal vesicles (Gunnersen et al., 1996, Yki-Jarvinen et al., 1983). In mice (Samuel et al., 2003) and rats (Gunnersen et al., 1995) relaxin is expressed in testicles and prostate. In man the expression of H3 RLX has been demonstrated in testicles but the biological role is still unknown (Liu et al., 2003).

Non-reproductive tissues

RLX is expressed not only in reproductive organs. By RT-PCR it has been demonstrated that, both in atrial and ventricular portion of the human heart, small quantities of H1 and H2 RLX are expressed (Dschietzig et al., 2001). In mice, instead, relaxin mRNA is present in the brain, lung, kidney, liver, thymus, spleen and heart (Bathgate et al., 2002; Du et al., 2003; Samuel et al., 2004); in rats, it is present in the heart, brain, kidney, pancreas and liver (Osheroff e Ho, 1993; Gunnersen et al., 1995). The expression of H3 RLX in human is located essentially in the brain, but it has been demonstrated also in lymphnodes, spleen, and thymus (Bathgate et al., 2002); in mice instead it has been found in the brain (Bathgate et al., 2002), in the spleen, thymus, lung, heart, kidneys and liver (Bathgate et al., 2002; Samuel et al., 2003; Samuel et al., 2004); in rats, it is present in the brain and in the heart (Kompa et al., 2002; Samuel et al., 2004).

Relaxin family peptide receptors (RXFPs)

For numerous years, researchers have tried to identify RLX receptors; however, the structural resemblance between this hormone and insulin had led to the assumption that the two molecules shared similar receptors with tyrosine-kinase activity. Instead, in 2002 it was discovered that RLX is able to activate two receptors, until then orphans, belonging to the LGR (leucine-rich repeat-containing G protein-coupled receptors) family, namely LGR7 and LGR8 (Hsu et al., 2002; Rosenkranz et al., 2002). These receptors, that shared approximately 60% of the amino acid residues, have been renominated respectively Relaxin Family Peptide Receptors 1 e 2 (RXFP1 and

RXFP2) (Bathgate et al., 2006) A further confirmation of this important discovery derives from the fact that the inhibitors of the activation of G proteins abolish the response of target cells to RLX (Barstch et al., 2001). More recently, other G protein-coupled receptors of the relaxin family have been discovered: GPCR135 and GPCR142 (Liu et al., 2003), respectively renominated RXFP3 e RXFP4.

RXFP1 is the main receptor and it is the most specific for H2 RLX, however it binds also H3 RLX, it is expressed both in the reproductive apparatus during pregnancy and in the heart, brain, kidney and lung (Bathgate et al., 2003).

RXFP2 instead binds INSL3 peptide, but it is able to bind with less affinity also H1 and H3 RLX (Hsu et al 2002; Sudo et al., 2003).

RXFP3 and RXFP4 bind relaxin 3 (Liu et al., 2003). RXFP3 is highly expressed in the brain of rats (McGowan et al., 2006) and represents the endogenous receptor for relaxin 3.

RXFP4 is selectively activated by relaxin 3, even if with low affinity, and it has been located in various tissues, namely brain, kidney, testicles, thymus, placenta, prostate, salivary glands, thyroid and colon (Liu et al., 2003); recently it has been discovered that it represents the endogenous receptor for ISLN5 (Liu et al., 2003).

RXFP1 structure

RXFP1 is constituted in its N-terminal region by a low density class A lipoprotein module (LDLa), followed by two cysteine rich regions, separated by a multiple repetition of leucine (Multiple leucine-rich repeats LRR). The ectodomain is connected to a region formed by seven trans-membrane domains, followed by a C-terminal region (Figure 2). Episodes of alternative splicing on the region that codifies for the ectodomain generate the receptor's isoforms (Bathgate et al., 2006).

Exploiting RXFP1 chimeras, two binding sites for RLX have been identified: one with more affinity in the extracellular region, the other with low affinity, placed at the trans-membrane domain. The bond receptor-ligand takes place through synchronized chelation of the two arginine B13 e B17 present in RLX, due to the couple of aspartic acid and glutamic acid of the receptor. This bond is stabilized by the hydrophobic interaction that isoleucine B20 of the hormone carries out with the residues of tryptophan, isoleucine and leucine of the LRR domains on the receptor (Büllesbach et al., 2005). The receptor, once the binding has taken place with the RLX, faces a conformational change that favors the contact of the ligand with the transmembrane domain. This

new conformation determines, through the LDLa domain, the activation of the adenylate cyclase protein (AC) and the consequent production of cyclic AMP (cAMP) (Scott et al., 2006).

Of interest, there is approximately 85% sequence homology between rat, mice and human RXFP1 (Scott et al., 2004).

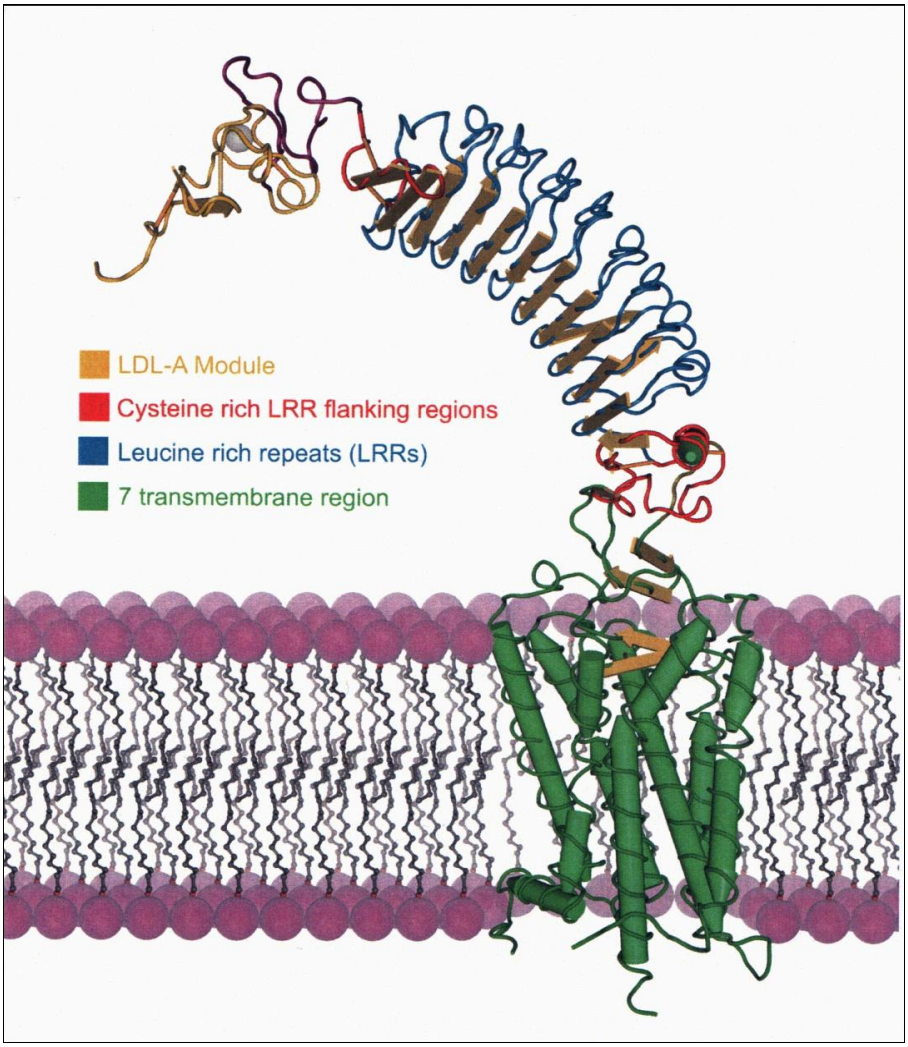

Fig.2 Schematic representation of the RXFP1 receptor (Bathgate et al., *Pharmacol Rev*, 58:7-31, 2006)

RXFP1 expression pattern

Since its discovery, RXFP1 transcripts have been found in several reproductive organs and non-reproductive organs known to be involved in RLX physiology (Sherwood et al., 2004). Hsu and colleagues demonstrated that in humans the RXFP1 transcript is present in the heart, brain, kidney, testis, placenta, uterus, ovary, adrenal, prostate, skin, liver and lung (Hsu et al., 2002). In rodents a similar expression pattern was seen including the heart, nipple, oviduct, small intestine, colon, thymus and spleen (Hsu et al., 2000; Krajnc-Franken et al., 2004; Scott et al., 2004)

RXFP1 dimerization as a de-activation mechanism

The regulation of RXFP1 has been shown to be similar to numerous GPCRs including the receptors for the thyroid-stimulating hormone, luteinizing hormone, and follicle-stimulating hormone (Svendsen et al., 2009), particularly in terms of their dimerization properties. RXFP1 was consistently shown to exist as a homo-and heterodimer receptor complex on the plasma membrane after being synthesized (Kern et al., 2008; Svendesen et al., 2008).

RXFP1 homodimers are often predominantly formed in the endoplasmic reticulum before being translocated to the cell membrane, thus implying that RLX receptor exists as a constitutive functional dimer once it reaches the cell surface (Kern et al., 2008; Svendsen et al., 2008). This suggests that the expression of RXFP1 at the cell surface is highly regulated by a number of factors during receptor synthesis and maturation, to ensure the smooth trafficking of the homodimer receptor complex to the plasma membrane. On the other hand, RXFP1 heterodimers are formed when RXFP1 interacts with other GPCRs that are co-expressed within the same cellular membrane. The dimerization of RXFP1 can occur either in the absence or presence of ligand binding to the receptor and receptor dimers are evident throughout the bioactivity of RFXP1 (Svendsen et al., 2008a; Svendesen et al., 2008).

Two intriguing consequences of RXFP1 dimerization are that i) it increases the functional range of RLX (over a broader range of concentrations), leading to the activation of different signaling pathways (Shymko et al., 1997); and ii) it leads to a “negative co-operativity” resulting in a rapid acceleration in the dissociation rate of RLX at the receptor as its concentration increases, which in turn causes lower physiological response to RLX at higher concentrations (Svendsen et al., 2008; Svendsen et al., 2009).

RLX signaling

In THP-1 (human acute monocytic leukemia) and HEK293T (human embrionic kidney) cell lines, it has been demonstrated that the binding of RLX to RXFP1 determines two mechanisms successive in time: during the first 1-2 minutes, through the α subunit of G protein, there is the activation of adenylate cyclase (AC), with the consequent increase of the intracellular cAMP levels. The following mechanism, within 10-20 minutes from the stimulation, foresees instead the activation, mediated by βγ subunit of G protein, of phosphatidylinositol (PI) 3-kinase (PI3K). The production of phosphatidylinositol-3-phosphate by PI3K determines the translocation to the membrane of protein kinase Cζ (PKCζ) that phosphorylates AC, increasing the level of intracellular cAMP (Nguyen and Dessaur, 2005). The transduction pathway that involves PI3K could be triggered also through tyrosine kinase activated by the same RXFP1 receptors (Bartsch et al., 2001).

RLX is also able to increase $NO^{\bullet}$ production, modulating the activation and expression of inducible NO synthase (iNOS or NOSII) and of endothelial-type NO synthase (eNOS or NOS III) (Baccari & Bani, 2008). The signaling transduction system involves PI3K, through which protein kinase B (PKB or Akt) is activated. The latter in turn activates eNOS, through the phosphorylation at serine 1179. An alternative mechanism, instead, is based on the increase of the cAMP level, with consequent activation of protein kinase A (PKA). The rise in intracellular cAMP level also activates cAMP-responsive element-binding protein 1 (CREB) to cause changes in gene expression for various proteins (Tanq et al., 2005) including iNOS (Nistri and Bani, 2003). H2 RLX and porcine relaxin-1 have been also found to bind and activate human nuclear glucocorticoid receptors in different human cell lines, such as macrophages, HeLa endometrial cancer cells and embryonic cells (Dschietzig et al., 2004) (Figure 3).

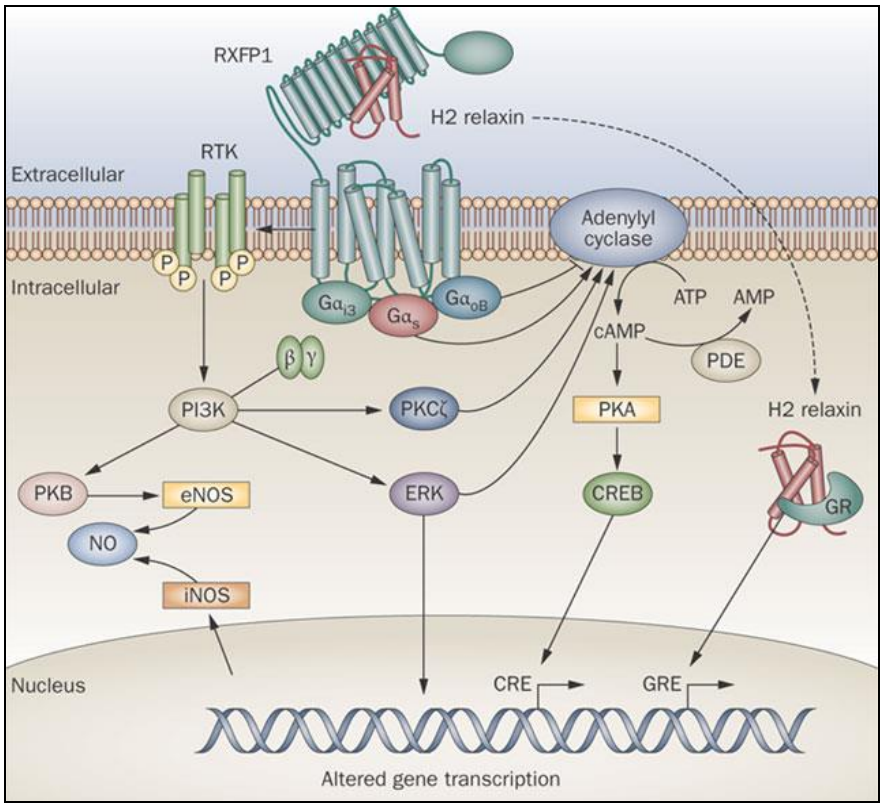

Fig.3 Signaling pathways activated by the RLX receptor RXFP1 (Du et al., *Nat Rev Cardiol* 7:48-58, 2010)

Phenotype of RLX deficient rodents

In vivo studies performed using antibodies that neutralize RLX (MCA1), have demonstrated how the hormone is able to carry out a trophic function for the female reproductive organs and the mammary apparatus (necessary for child birth and breast feeding). MCA1-treated rats show both a compromised development of the nipple (Kuenzi and Sherwood, 1992) and an under-development of the vagina and uterine cervix, with a high density of collagen fibers and less blood vessels in the stroma (Zhao and Sherwood, 1998). The treated rats are also subject to non-vital deliveries and the cubs have smaller dimensions in respect to the relative control (Guico-Lamm and Sherwood, 1988). The minor development of the organs seems ascribable to an accumulation of collagen and to its diminished re-modelling, usually mediated by RLX (Zhao et al., 1999).

The use of RLX knockout mice (RLX -/-) has allowed to confirm the results obtained with the

MCA1 antibodies, validating the concept that RLX is necessary for the growth and differentiation of the mammary gland and the maturation of the uterus cervix; it is necessary, furthermore, to relax the birth channel and to inhibit contract activity of the uterus, (Skott and Carter, 2002).
Also, male RLX -/- mice show underdevelopment in the sexual organs: inadequate dimension of the prostate, testicles and epididymis (Samuel et al., 2003).

Biological effects of RLX

RLX has a long history in the reproductive field and a relatively short history in the non-reproductive field, as extensively reported in previous reviews (Bani 1997, Bathgate 2006, Goldsmith et al., 1995; Sherwood 2004; Weiss et al., 2001). The use of recombinant H2 RLX and availability of RLX- and RXFP1-deficient mice have allowed researchers to better understand the effects of RLX in both the reproductive and non-reproductive organs. A detailed review of the non-cardiovascular actions of RLX is beyond the scope of this thesis, but a brief summary of the main findings is appropriate before shifting back the focus to the effects of RLX in the cardiovascular system.

Effects on the reproductive system

The first biological activity attributed to RLX is the lengthening of the interpubic ligament and the softening of the tissues of the cervix and vagina, which have an effect of facilitating the passage of the fetus at birth.
RLX, in synergy with ovarian steroids, is required to promote the growth of the mammary gland (Sherwood 1994; Bani 1997) and to induce a normal development of the nipple (Kuenzi et al., 1995). At the uterine level, in laboratory mammals, RLX can inhibit the spontaneous contractile activity of the myometrium (Krantz et al., 1950) thereby facilitating the accommodation of the conceptus (Downing and Hollingsworth, 1993).
The regulation of RLX production and secretion is species-dependent (Sherwood 2004; Bathgate et al., 2006). Interestingly, the profile serum RLX levels in humans greatly differs to that of rodents, whereby: i) serum RLX levels are highest during the normal menstrual cycle and the first trimester

of pregnancy, which subsequently decline throghout pregnancy; ii) maximal serum RLX levels reach 1-2 ng/ml during pregnancy, which is very much lower than the 150-200ng/ml of circulating RLX that peak around late gestation in pregnant rodent (Sherwood and Crnekovic 1979); and iii) human RLX does not accumulate in the storage granules of the lutual cells (Eddie et al., 1986; Bell et al., 1987). Human RLX has thus been implicated in playing a crucial role in preparing the uterus for embryonic implantation rather than maintaining pregnancy and facilitating childbirth (Van Der Westhuizen et al., 2007).

Although RLX is often associated as a female pregnancy-related hormone, it is also produced in males but in smaller quantities by the prostate (primarily within the glandular epithelium) and/or testes, which is subsequently secreted into the seminal fluid to increase spermatozoa motility to enhance male fertility (Essig et al., 1982; Essig et al., 1982; Sokol 1989). Morover, RLX in the seminal fluid can act on the female uterine receptor to promote thickening of the endometrium and angiogenesis to prepare the uterus for embryo implantation (Weiss 1989).

Effects on the non-reproductive system

Kidney

RLX has been shown to promote renal vasodilatation and hyperfiltration, while reducing the myogenic reactivity in small renal arteries of both pregnant and non-pregnant rats (Conrad et al., 2004; Danielson et al., 2003; Novak et al., 2001). It was found that exogenous RLX could increase effective renal plasma flow and glomerular filtration rate, attenuate the renal circulatory response to Angiotensin II and reduce plasma osmolality regardless of sex (Danielson et al., 2000; Danielson et al., 1999). This work was extended to humans. The renal effect of H2 RLX was examined in both male and female and RLX was found to increase plasma flow confirming that RLX is indeed one of the renal vasodilatory factors in human pregnancy (Smith et al., 2006).

Lungs

Bani and co-workers found that RLX possesses anti-asthmatic properties by using ovalbumin-sensitized guinea pigs (Bani et al., 1997). RLX was able to reduce the severity of respiratory abnormalities, histological alterations, mast cell degranulation and leukocyte infiltration; additionally, the hormone was able to promote dilatation of alveolar blood capillaries and reduce the thickness of the air-blood barrier (Bani et al., 1997). Later on, RLX was demonstrated to inhibit

airway remodeling and hyperresponsiveness in models of allergic airways disease (Royce et al., 2009) and improve pulmonary hypertension (Tozzi et al., 2005).

Brain

The brain is another target organ for RLX as evidenced by increases in the presence of RLX and its receptors in numerous regions of the brain involved in a broad range of sensory and autonomic neural functions. Experimental studies suggest central effects of circulating RLX include: timing of parturition, plasma osmolality, milk ejection and release of oxytocin, autonomic control of fluid homeostasis and release of vasopressin (Dayanithi et al., 1987; Sortino et al., 1989; Summerlee et al., 1998; Summerlee et al., 1984). Additionally, intracerebroventricular RLX administration impairs memory consolidation and stimulates the intake of food and water (Hornsby et al., 2001; Ma et al., 2005; McGowan et al., 2005; McGowan et al., 2006; Summerlee et al., 1998).

Connettive tissue and fibrosis

In the first years of the ‘80s, Too and collaborators (Too et al., 1984), through *in vitro* experiments on rat ovarian granulosa cells, observed that RLX stimulates the release of matrix metalloproteinase (MMPs). Through *in vivo* studies on female rats, it has been noticed that treatment with porcine RLX decreases the amount of collagen in the uterine cervix (Downing and Sherwood, 1986). Further studies carried out on dermal fibroblasts have demonstrated that RLX, increasing the expression of MMPs, down-regulates the production of tissue inhibitors of MMPs (TIMP) and the secretion of collagen (Unemori and Amento, 1990). In this same model, RLX was able to inhibit the production of collagen stimulated by TGF-β (Unemori and Amento, 1990).

By *in vitro* experiments on human uterine cervical fibroblasts, it has been demonstrated that RLX determines an increase in the espression of specific MMPs: MMP-1, MMP-2 e MMP-3 (Palejwala et al., 2001). RLX, through the modulation of the expression of MMPs and the regulation of secretion of collagen, promotes its remodeling, thus contrasting the process of fibrosis. This has been demonstrated both *in vitro* and *in vivo* not only in the female reproductive apparatus, but also in other disctricts such as heart, skin, lungs, liver and kidneys. (Unemori and Amento, 1990; Unemori et al., 1996; Du et al., 2003; Garber et al., 2003; Samuel et al., 2003)

Regarding skin, it has been demonstrated that RLX is able to modulate the secretion of collagen from dermal fibroblasts (Unemori and Amento, 1990) and to decrease collagen deposition in rat and mouse models of fibrosis (Unemori et al., 1993). Furthermore, according to a study relative to a clinic trial carried out on a cohort of 68 patients with scleroderma, the treatment with RLX is associated with a reduced cutaneous thickness (Seibold et al., 2000).

Regarding the lung, the administration of RLX decreases collagen deposition in a mice model of fibrosis (Unemori et al., 1996). Moreover, a synthetic peptide with RXFP1-agonist activity was able to carry out an antifibrotic action in a murine model of bleomycin-induced pulmonary fibrosis (Pini et al., 2010). In a rat model of liver cyrrhosis, RLX decreases collagen deposition and shows an antifibrotic action (Williams et al., 2001). Similarly, in a rat model of kidney fibrosis, RLX was able to inhibit the activation of fibroblasts (Hegg et al., 2005) and carries out an antifibrotic action (Garber et al., 2001) (Lekgabe et al., 2005).

RLX AND THE CARDIOVASCULAR SYSTEM

In the last 30 years, RLX has been validated as a *bona fide* cardiovascular hormone (Bani 1997; Dschietzig et al., 2002; Nistri et al., 2007). Indeed, convincing evidence has been provided that RLX is produced by the heart, it possesses specific receptors at the cardiac level and it acts on the myocardium, blood vessels and blood cells.

Myocardial RLX expression

In 1994 a study reported that when cardiomyocytes derived from the atria of neonatal rats were cultured in monolayers, immunoreactive RLX was detected in the conditioned medium (Taylor and Clark 1994). This study provided the first evidence that RLX is secreted by cardiomyocytes and opened the possibility that it may act through a paracrine route to regulate cardiovascular function.
Since then, both H1 and H2 RLX, but not H3 RLX, have been found to be expressed in human atria and ventricles and, furthermore, the expression was demonstrated to increase dramatically under pathological conditions, such as cardiomyopathy and heart failure (Dschietzig et al., 2001). Expression of the H1 and H2 RLX mRNA was also evident in mammary arteries and saphenous veins and was believed to be produced by cardiomyocytes and interstitial cells (Dschietzig et al., 2001).
In accordance with findings in humans, relaxin 3 was found to be expressed in the atrial and ventricular myocardium, in atrial and ventricular myocytes and fibroblasts in addition to vascular smooth muscle (Kompa et al., 2002; Samuel et al., 2004). Separate *in vitro* studies using isolated rat

hearts confirmed that, along with elevation of ventricular filling pressure mimicking the hemodynamic changes associated with heart failure, there was an up-regulation of ventricular RLX expression (Dschietzig et al., 2001).

Myocardial RXFP1 expression

In 1992, Osheroff and colleagues reported the existence of high–affinity binding sites for [^{32}P]-labeled synthetic H2 RLX in the rat cardiac atria, from as early as day one after birth up until adulthood, thereby indicating that the heart is a target organ for RLX (Osheroff et al., 1992). Subsequent to the discovery of the RXFP1 receptor in 2002, transcripts for this receptor were identified in the heart of rats (Hsu et al., 2002; Kompa et al., 2002; Samuel et al., 2004), mice (Krajnc-Franken et al., 2004) and humans (Hsu et al., 2002). In particular, the expression of RXFP1 mRNA was determined in rat atria and left ventricles (Kompa et al., 2002) and in rat cardiomyocytes (Nistri et al. 2012; Moore et al., 2014).

Chronotropic effects

Numerous *ex vivo* studies have shown that RLX exerts a positive chrontropic effect both on the whole perfused heart and on the isolated right and left atrium as well as on the ventricular portion (Ward et al., 1992; Kakouris et al., 1992; Han et al., 1994; Tan et al., 1998; Conrad et al., 2004). The positive chronotropic effect attributed to RLX was also confirmed by *in vivo* studies on rats (Ward et al., 1992; Kakouris et al., 1992; Han et al., 1994; Tan et a.,l 1998; Conrad et al., 2004).

RLX is able to influence cardiac frequency through the increase of intracellular cAMP, which in turn activates a cAMP-dependent protein kinase. The latter mediator determines a rise in the concentration of intracellular calcium, with consequent increase in L-type current mediated by calcium channels (Han et al., 1994).

Experiments conducted on rat isolated atria have highlighted that the positive chronotropic effect provoked by RLX results more efficient than that produced by known chronotropic substances such as endothelin-1, angiotensin-II, isoproterenol, adrenalin, histamine and serotonin (Kakouris et al., 1992).

Vasodilatatory and neoangiogenetic effects

The local and systemic administration of RLX produces an immediate vasodilation. This vasodilatatory effect has been first observed in the ‘80s in the mammary gland of mice to which porcine RLX had been administered (Bani and Bigazzi, 1984). The effect of the hormone has been observed not only in the reproductive apparatus, but also in mesocaecum (Bigazzi et al., 1986), in the coronary arteries of rats and guinea pigs (Bani-Sacchi et al., 1995) in the pulmonary microcirculation of guinea pigs (Bani et al., 1997), in the pigeon crop sac (Bigazzi et al., 1998), in the rat kidney (Novak et al., 2001) and in rat liver sinusoids (Bani et al., 2001). In particular, the vasodilatation induced by RLX in the coronary arteries results in a significant increase of blood flow which, in rat and guinea pig models, was found to be 100 fold more intense than that produced by acetylcholine and 1000 fold more intense than sodium nitroprusside (Bani-Sacchi et al., 1995; Bani et al., 1998).

The RLX-induced vasodilatation appears to depend on the stimulation of endogenous generation of the potent vasorelaxant agent $NO^{\bullet}$ by vascular cells (Bani et al., 1998; Danielson et al 1999; Failli et al., 2002). In particular, RLX was found to increase the expression and activity of NOS II (iNOS) in endothelial and vascular smooth muscle cells (Bani et al., 1998; Failli et al., 2002). RLX could also indirectly induce $NO^{\bullet}$ generation through an endothelin-dependent pathway. In fact, RLX promotes secretion of collagenases, which causes proteolytic cleavage of big endothelin-1 into bioactive endothelin. In turn, endothelin could bind to and activate endothelin-B receptors, which are up-regulated by RLX (Conrad 1999) thereby inducing constitutive NOS III (nNOS) activation and NO release (Conrad 1999; Unemori 1999).

Besides vasodilatation, RLX can also induce angiogenesis in some target organs, as suggested by the pioneer findings by Hisaw (1967) using partially purified RLX. In human endometrial cells *in vitro,* RLX induces the production of potent angiogenic molecules such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Unemori et al., 1999). In a rat model of chronic myocardial infarction, systemic infusion of RLX potentates bFGF mRNA expression in the peri-infarct region by both cardiomyocytes and fibroblasts (Lewis et al., 2001). Similarly, in a swine model of post-infarcted heart, local production of RLX by RLN2 gene-transfected myoblasts grafted into the post-ischemic myocardium, causes a significant increase in microvessel density and over-expression of VEGF mRNA by the host cardiac cells (Formigli et al., 2007).

Thus, RLX, by promoting vasodilation and, at least in some conditions, angiogenesis could influence the perfusion of many target organs.

Anti-inflammatory and anti-thrombotic effects

Through the modulation of the production of $NO^{\bullet}$, RLX also performs anti-inflammatory effects, as it can inhibit: i) the endothelial adhesiveness to neutrophils in pro-inflammatory conditions *in vitro* by down-regulating early- and late-phase endothelial cell adhesion molecules (Nistri et al., 2003); ii) the activation of neutrophils challenged *in vitro* with inflammatory mediators, by reducing oxidative burst, ROS generation and chemotaxis (Masini et al., 2004); iii) the activation of circulating basophilis, thereby reducing the release of histamine and of the other mediators of the inflammation (Masini et al., 1997); iv) the release of histamine by perivascular mast cells in guinea pig and rat models of inflammation (Masini et al., 1994; Bani et al., 2002; Nistri et al., 2008).
RLX is able to regulate blood homeostasis also influencing the number and hemostatic function of platelets. From studies carried out in rabbits and rats, RLX was shown to stimulate platelet $NO^{\bullet}$ production, to inhibit platelet aggregation and to decrease the number of circulating platelet, impeding their release from the megakaryocytes (Bani et al., 1995). RLX also promotes fibrinolysis, stimulating the release of tissue plasminogen activator (Qin et al., 1997; Wang-Lee et al., 1998).

Anti-fibrotic effects

There are numerous studies that confirm that RLX is able to contrast cardiac fibrosis. In a model of myocardial fibrosis obtained in rats with spontaneous hypertension, it was demonstrated that RLX normalizes the content of collagen and inhibits the proliferation and differentiation of fibroblasts (Lekgabe et al., 2005). In a model of cardiac fibrosis carried out in transgenic rats overexpressing the adrenergic β2 receptor, the treatment with engineered adenovirus for the production of RLX determines a significant decreasing of interstitial collagen in the left ventricle (Bathgate et al., 2008). Furthermore, in a rat model of cardiomyopathy, the treatment with H2 RLX promotes an improvement of collagen accumulation in the left ventricle, decreases myofibroblast activation and increases MMP matrix degradation (Samuel et al., 2008). Recently, RLX has demonstrated to be able to favourably influence the process of fibrotic healing in swine and rodent models of myocardial infarction (Formigli et al., 2007; Bonacchi et al., 2009; Samuel et al., 2011). In these models, RLX determines a significant reduction of TGF-β1 expression, myofibroblast differentiation and cardiomyocyte apoptosis in addition to a promotion of MMP levels and de novo blood vessel growth.

Moreover, treatment with RLX of rat cardiac fibroblasts in culture causes decrease in the production of collagen, inhibition of fibroblasts differentiation into myofibroblasts and increase in MMP secretion. The hormone is also able to contrast the growth- and differentiation-inducing effects of TGF-β on cardiac fibroblasts (Samuel et al., 2004). In this context, it has been recently demonstrated that RLX is able to inhibit TGF-β-induced transition of cardiac fibroblasts to myofibroblasts by a mechanism involving the Notch-1 signal pathway (Sassoli et al., 2013).

Myocardial regeneration and remodelling

The many cardiotropic properties of RLX could underlie and justify future research to test its possible therapeutical use in stem cell grafting for myocardial regeneration of post-infarcted heart, possibly favouring its colonization with precursor cells able to settle into the scar, to establish functional relationships with the residual cardiomyocytes, and to differentiate *in situ* into contractile elements functionally integrated with the surrounding myocardium.

An *in vitro* study, conducted on co-cultures of skeletal myoblasts and adult cardiomyocytes demonstrates how treatment with H2 RLX favors the intercellular coupling between cardiomyocytes and between cardiomyocytes and myoblasts not only by the formation of new junctions but also by potentiation of electrical coupling of the pre-existing junctions (Formigli et al., 2005). Similar results have been obtained on cardiomyocyte cultures in the presence of myoblasts genetically engineered to over-express H2 RLX (Formigli et al., 2009).

A pioneer study conducted on a pig model of chronic myocardial infarction has demonstrated that the inoculum of mouse skeletal myoblasts genetically engineered to express RLX, induced a straightforward functional improvement of the post-infarcted heart, favoring the re-modelling of the extracellular matrix and increasing the microvascular density (Formigli et al., 2007).

Similarly, in a rat model of chronic myocardial infarction, the inoculum of mouse skeletal myoblasts genetically engineered to express RLX and treatment with exogenous RLX improved the main echocardiographic parameters of cardiac function, increased myocardial viability, decreased cardiac sclerosis and myocardial cell apoptosis and increased microvascular density in the post-infarction scar tissue (Bonacchi et al., 2009).

Recently, it has been demonstrated that RLX specifically acts on immature cardiomyocytes by promoting their proliferation and maturation. These notions suggest that RLX may be an endogenous regulator of cardiac morphogenesis during pre-natal life and could participate in heart regeneration and repair (Nistri et al., 2012)

Effects on I/R injury

To study the possible benefits of RLX in the treatment of myocardial infarction, experimental models of I/R injury were used, both *ex vivo* on isolated and perfused hearts and *in vivo* by the temporary closure of the left anterior descending coronary artery. The e*x vivo* studies conducted on guinea pig hearts, adding porcine RLX to the perfusion liquid 30 min before coronary occlusion, evidenced that the hormone was able to increase the coronary blood flow already during ischemia and to a greater extent at reperfusion. In this model, RLX was able to blunt the severity of ischemia and facilitate the removal of inflammatory mediators and ROS during reperfusion (Masini et al., 1997). In an *in vivo* rat model of acute myocardial infarction, similar results have been obtained by administering intravenous porcine RLX, 1 h before the induction of ischemia (Bani et al., 1998). In this model, a smaller extension of the damaged myocardial area and a diminished production of ROS were observed upon RLX treatment. At the ultrastructural level, aspects of cardiomyocyte and endothelial cell necrosis were not detected, while the cardiomyocytes of the ischemic area showed a moderate grade of signs of contractile dysfunction, e.g. myofibril hypercontraction and mitochondrial calcium accumulation (Bani et al., 1998). More recently, Zhang and collaborators (Zhang et al., 2005) have evaluated the effects of relaxin 3 in a rat ischemic heart model obtained by functional overload through the β-adrenergic agonist isoproterenol. The induction of cardiac damage causes an increase of relaxin 3 level both in the myocardium and in the plasma, while the exogenous administration of the hormone determines a reduction of ROS-induced myocardial injury, inflammatory cell infiltration and myocardial fibrosis.

These studies have demonstrated that RLX can exert a protective action towards the heart undergoing hypoxic damage when administered before the ischemic insult, suggesting a possible preventive therapeutic use of RLX, or RLX analogues, in myocardial infarction. Subsequently, further studies were conducted to evaluate if RLX could bring therapeutic benefits even if administered after the ischemic event, thus trying to bridge the gap between experimental model and clinic reality, where the possible therapies can be carried out upon hospitalization or even during surgical re-opening of the occluded coronary artery (coronary angioplasty), e.g. after the ischemia. With this intent, Perna and collaborators (Perna et al., 2005) have carried out experiments on a pig model of acute myocardial infarction in which H2 RLX was administered in the coronary artery after 30-min. ischemia at the moment of reperfusion. In this model, RLX caused a marked, dose-related reduction of the main serum markers of myocardial damage, namely myoglobin, CK-MB and troponin T, as well as of the metabolic and histopathological parameters of myocardial inflammation and cardiomyocyte injury and apoptosis. These effects of RLX resulted in increased

myocardial salvage, and improved ventricular contractile performance.

On the above basis, it is possible to hypothesize a therapeutic use of RLX in ischemic cardiomyopathy. The validity of such hypothesis is confirmed by the findings of a recent clinical trial on patients with acute heart failure, where the administration of H2 RLX (Serelaxin, Novartis) has demonstrated a good effectiveness in prolonging life expectancy in patients treated with the hormone in respect to the group treated with placebo. In particular, the completion of phase II and phase III trials in as many as 1400 patients has clearly demonstrated that RLX improved dyspnea, various markers of cardiac and renal damage/dysfunction and lowered the number of re-hospitalization and deaths (by 37%) in a 180 day follow-up period. (Teerlink et al. 2009; Teerlink et al., 2013).

Intracellular mechanisms of RLX-induced protection against I/R injury

Based on the known signalling pathways downstream RXFP1, there are multiple mechanisms whereby RLX can induce protection to cardiac cells against I/R injury: among them, the Notch-1 pathway holds a pivotal place. Notch 1 is a transmembrane receptor that, once bound to its ligand, undergoes proteolytic cleavages managed sequentially by ADAM 10 metalloprotease and the γ-secretase complex. These events allow the release of the intracellular domain of Notch (Notch-ICD) that translocates to the nucleus to control transcription of specific target genes (Brou 2009).

The relationship between RLX and Notch-1 is supported by the demonstrations that Notch-1 is involved in ischemic preconditioning and postconditioning of the heart (Yu and Song, 2013; Zhou et al., 2013) and in the reduction of I/R-induced cardiac nitroxidative stress (Pei et al., 2013). Of note, the involvement of Notch-1 in target cell response to RLX has been recently demonstrated: indeed, RLX has been shown to antagonize the TGF-β-induced transition of cardiac fibroblasts to myofibroblasts by activation of Notch-1 pathway (Sassoli et al., 2013).

Aim-1

The aim of the first part of this thesis is to investigate the possible protective actions of RLX on cardiomyocytes using an *in vitro* model of I/R – i.e hypoxia followed by reoxygenation (H+R) - and to investigate the intracellular mechanisms by which RLX may exert cardioprotection, paying special attention to the Notch-1 pathway.

Materials and Methods

Ethical statements

Animal handling and use complied with the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986; 86/609/EEC) and were approved by the Committee for Animal Care and Experimental Use of the University of Florence. The ethical policy of the University of Florence conforms to the Guide for the care and use of laboratory animals of the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance No. A5278-01). The animals had free access to food and water and were housed on a 12 h light/dark cycle at 22°C room temperature. The experiments were designed to minimize pain and the number of animals used. Sacrifice was carried out by decapitation.

Cell culture and treatments

H9c2 embryonic rat myocardium-derived cells, a well characterized and widely used cell line to study myocardial cell ischemia (Hescheler et al., 1991), were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma- Aldrich, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 250 U/ml penicillin G and 250 μg/ml streptomycin (Sigma-Aldrich), in a humidified atmosphere with 5% CO_2 at 37 °C. Primary cultures of mouse ventricular immature cardiomyocytes were prepared from hearts of 1-day old newborn CD1 albino mice (Harlan, Correzzana, Italy), as described (Nistri et al., 2012). Briefly, hearts were excised, the ventricles minced and digested at 37°C for 45 min in calcium-free

HEPES-buffered Hanks' solution, pH 7.4, containing 100 μg/ml type II collagenase (Invitrogen). To reduce the harvest of nonmyocardial cells, the tissue lysate was filtered through a 70μm cell strainer (Millipore, Billerica, MA, USA) and preplated for 1 h. The myocyte-enriched cells remaining in suspension were seeded on collagen-precoated multi-well plates. Cardiomyocytes were cultured in DMEM containing 10% horse serum, 5% fetal bovine serum, 2 mM glutamine, 250 U/ml penicillin G and 250 μg/ml streptomycin. In previous studies, these cells were characterized morphologically, immunophenotypically and electrophysiologically (Nistri et al., 2012; Formigli et al., 2009) and were shown to express the specific RLX family peptide receptor-1 (RXFP1) (Formigli et al., 2009).

H9c2 cells and primary cardiomyocytes were subjected to H+R, simulated *in vitro* by substrate starvation plus hypoxia followed by reoxygenation as described (Zhang et al., 2012) with minor modifications. The cells were incubated in DMEM with no serum or glucose and placed in a hypoxic chamber saturated with a 0,1% O_2 , 5% CO_2, ≈95% N_2 gaseous mix, humidified and warmed at 37°C, for 7 h. At end hypoxia, the cells were reoxygenated for 2 h by incubation in normoxic conditions in glucose-containing, serum-free DMEM. Control normoxic cultures were also prepared. Cells were treated or not with human recombinant H2 RLX (RLX) (17 nmol/l), kindly provided by the RRCA Relaxin Foundation (Florence, Italy), administered in two different ways: in some experiments, RLX was added preventatively, i.e. 24 h before hypoxia and maintained for the whole duration of H+R (RLX+ H. and RLX+H+R); in other experiments, RLX was added at reoxygenation, concurrently with the peak of ROS generation (H+RLX+R). The noted RLX dose was chosen as the most effective on the basis of preliminary dose-finding experiments (5-50 nmol/ml) performed on H9c2 cell viability.

As control for specificity of the RLX effects on cell viability, some experiments (MTT assay) were performed using inactivated RLX (iRLX, 17 nmol/l) in the place of authentic RLX. Inactivated RLX was obtained by blockade of functional arginine residues by reaction with cyclohexanedione followed by dialysis of the unbound reagent against distilled water, according to the method of Büllesbach and Schwabe (Büllesbach and Schwabe, 1988). To test the effective lack of bioactivity of iRLX, we measured cAMP generation in human THP-1 cells constitutively expressing RXFP1, as described (Bani et al., 2007). These findings confirm that, at variance with authentic RLX, iRLX did not induce any cAMP rise in THP-1 cells. The effects of iRLX were evaluated at reperfusion, when authentic RLX showed the highest cell protection.

To investigate the role of the Notch-1 pathway in the mechanism of action of RLX, H9c2 cells were treated with N-[N-(3,5 difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT; 5 μM, Sigma-Aldrich), a highly active γ-secretase inhibitor which blocks the generation of Notch intracellular domain (Notch-ICD), the active proteolytic fragment of Notch-1 receptor that mediates

its biological effects. At the end of the treatments, the cell cultures were photographed under a phase contrast inverted microscope.

Reverse Transcription (RT) and real-time PCR

To evaluate if H9c2 cells express RXFP1 mRNA, one μg of total RNA, extraction with TRIzol Reagent (Invitrogen), was reverse transcribed and amplified with SuperScript One-Step RT-PCR System (Invitrogen). After cDNA synthesis for 30 min at 55°C, the samples were pre-denatured for 2 min at 94°C and then subjected to 38 cycles of PCR performed at 94°C for 15 s, alternating with 57°C for 30 s and 72°C for 1 min; the final extension step was performed at 72°C for 5 min. The following rat gene-specific primers were used: RXFP1 (NM_ 201417.1), forward 5'-CGG ATG GGA TCT CCT CTC TT-3'and reverse 5'-GCG TGC TTC CTG TAC TCT CC-3'. PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide.

To quantify bcl-2 and bax gene expression, 1 μg of total RNA was reverse transcribed with iScript cDNA Synthesis Kit (Biorad, Milan, Italy). Samples were incubated at 25°C for 5 min followed by 30 min at 42°C and 5 min at 85°C. Appropriate negative controls were carried out. Quantitative real-time PCR was performed using iTaq Universal SYBR Green Supermix (Biorad) on an ABI Prism 7900 HT detection system (Applied Biosystems Foster City, CA). PCR amplifications were performed in Optical 96-well plates (Applied Biosystems) on cDNA samples corresponding to a final RNA concentration of 10 ng. The relative quantitation values of targets were normalized to the endogenous GAPDH control gene. The primers used in the current study were chosen from previously published studies (Prasanna and Rasool, 2014): bcl-2 (NM_016993.1) forward 5'-GCT ACG AGT GGG ATA CTG G' and reverse 5'-GTG TGC AGA TGC CGG TTC A-3'; bax (NM_ 017059.2), forward 5'-CTG CAG AGG ATG ATT GCT GA-3' and reverse 5'-GAT CAG CTC GGG CAC TTT AG-3'and GAPDH (NM_017008.4), forward 5'- AAC GGC ACA GTC AAG GCT GA-3' and reverse 5'-ACG CCA GTA GAC TCC ACG ACA T-3'. Reaction conditions were as follows: 95 °C for 1 min., followed by 40 cycles at 95°C for 15 s alternating with 55°C for 1 min for bax and GAPDH or 57°C for 1 min for bcl-2. PCR amplifications were run in triplicate. Blank controls, consisting in no template (water) were performed in each run. Melting curves were carried out to confirm amplification of single sequences and absence of primer dimers. The results of the real-time PCR data were represented as Ct values, where Ct was defined as the PCR threshold cycle at which amplified product was first detected. Ct values were analysed using $2^{-\Delta\Delta Ct}$ comparative method.

Western blotting

After treatments, H9c2 cells were lysed in cold buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 1.5 mM, MgCl2, 2 mM Na2 EDTA, 1% Triton X-100), added with 10X Sigmafast Protease Inhibitor cocktail tablets (Sigma-Aldrich). Upon centrifugation at 13.000 g for 20 min at 4°C the supernatants were collected and the total protein content was measured spectrophotometrically using micro-BCA^{TM} Protein Assay Kit (Pierce, IL, USA). Forty μg of total proteins from cell lysates were electrophoresed by SDS–PAGE and blotted onto nitrocellulose membranes (Amersham, Cologno Monzese, Italy). The membranes were blocked with PBS containing 0.1% Tween (Sigma-Aldrich) and 5% bovine serum albumin (AT-PBS) (Sigma-Aldrich) for 1 h at RT and incubated overnight at 4°C with rabbit monoclonal anti-Notch-1 antibody (1:2000; Abcam) and rabbit polyclonal anti- GAPDH antibody (1:1000, Cell Signaling Technology), assuming GAPDH as control invariant protein. Specific bands were detected using rabbit peroxidase-labeled secondary antibodies (1:15.000; Vector, Burlingame, CA) and ECL chemiluminescent substrate (BioRad, Milan, Italy). Densitometric analysis of the bands was performed using Scion Image Beta 4.0.2 image analysis software (Scion Corp.) and the values normalized to GAPDH.

Trypan Blue viability assay

The trypan blue exclusion method was used to further assess cell viability. H9c2 cells ($5x10^4$/well) and primary cardiomyocytes ($3\text{-}5x10^4$/well, depending on the number of littermates and the overall yielding of the isolation procedure) were seeded in 24-well plates. At end treatments, the cells were gently harvested and mixed with 0.4% trypan blue solution (Sigma-Aldrich); the resulting cell suspension was counted under a phase contrast inverted microscope using a Burker chamber. The viable cells were expressed as percentage of the total counted cells.

MTT viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). H9c2 cells ($5\ x10^4$/well) and primary cardiomyocytes ($3\text{-}5x10^4$/well) were seeded in 24-well plates. At end treatments, MTT stock solution was added to each well and incubated for 4 h at 37°C. Dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The plate was gently shaken for 10 min. and was read at 550 nm on a plate reader. Optical density was assumed as indicator of mitochondrial activity and, indirectly, cell viability.

TUNEL assay

H9c2 cells were grown on glass coverslip and subjected to the different treatments. Cell death was studied with TUNEL assay for apoptosis, performed using a Klenow-FragEL^{TM} DNA fragmentation detection kit (Calbiochem, San Diego, CA), as reported in the manufacturer's instructions. Briefly, H9c2 cells were incubated with 15 mg/ml proteinase K for 5 min at room temperature. After rinsing in PBS, the cells were immersed in the Klenow Labelling Reaction Mixture containing deoxynucleotidyl transferase and biotin-labeled and unlabeled deoxynucleotides, and incubated at 37° C for 90 min in a humid atmosphere. Then, the cells were incubated with peroxidase-conjugated streptavidin for 30 min at room temperature and the signal was revealed with 3,3'-diaminobenzidine. Finally, nuclear counterstaining was achieved by Methyl green. Apoptotic nuclei were recognized by the presence of dark brown staining, at variance with those of viable cells, which instead appeared pale brown or green. TUNEL-positive nuclei were counted in five microscopic fields for each cell preparation. TUNEL apoptotic index was then expressed as relative percentage of TUNEL positive nuclei on the total number of methyl green-stained nuclei.

Immunohistochemical localization of nitrotyrosine

Nitrotyrosine (NT), an index of protein nitrosylation by harmful oxidants generated during inflammation, such as peroxynitrite ($ONOO^-$) was determined by immunocytochemistry as described previously (Cuzzocrea et al., 2001). H9c2 cells were grown on glass coverslip and subjected to the different treatments. The cells were fixed with formaldehyde for 10 min and then incubated with rabbit polyclonal anti-NT antibody (Upstate Biotechnology, Buckingham, UK; 1:118) at 4°C overnight. Immune reaction was revealed by goat anti-rabbit IgG conjugated with biotin (1:200; Vector Lab, Burlingame, CA, USA) followed by incubation with ABC complex (Vector Lab; 1:200). Negative controls were carried out by omitting the primary antibodies. Densitometric analysis of the intensity of NT was performed on digitized images using Scion Image Beta 4.0.2 in 20 regions of interest (ROI) of 100 μm^2 for each confocal stacks (at least 10).

Determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

8-OHdG levels, an indicator of oxidative DNA damage, were determined in H9c2 cells using the Highly Sensitive 8-OHdG Check (JaICA, Japan), according to the manufacturer's instructions. After treatments, the cells were collected with TRIzol Reagent (Invitrogen) and DNA was isolated according to the manufacturer instructions. Then DNA was subjected to enzymatic digestion with 10 IU of P1 nuclease (Sigma-Aldrich) in 10μL and incubated for 1 h at 37°C with 5 IU of alkaline

phosphatase (Sigma-Aldrich) in 0.4 M phosphate buffer, pH 8.8. All of the procedures were performed in the dark under argon. The mixture was filtered by an Amicon Micropure-EZ filter (Millipore), and 50 μl of each sample was used for 8-OHdG determination. The values are expressed as ng 8-OHdG/ng total DNA.

Transmission electron microscopy

After treatments, H9c2 cells were pelleted by centrifugation, fixed in 4% glutaraldehyde and 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol,Tokyo, Japan) at 80 KV

Confocal Immunofluorescence

H9c2 cells grown on glass coverslips were fixed with 0.5% buffered paraformaldehyde for 10 min at room temperature. After permeabilization with cold acetone for 3 min, the fixed cells were blocked with 0.5% bovine serum albumin (Sigma-Aldrich) and 3% glycerol in PBS for 20 min and then incubated overnight at 4°C with a rabbit monoclonal anti-Notch-1 antiserum (1:200, Abcam) recognizing both Notch-1 receptor and its activated form Notch- ICD. Immunoreactions were revealed by specific anti-rabbit Alexa Fluor 488-conjugated IgG (1:200; Molecular Probes, Eugene, OR) for 1 h at RT. Negative controls were carried out by replacing the primary antibody with non-immune serum; cross-reactivity of the secondary antibody was tested in control experiments in which primary antibodies were omitted. After washing, the immunolabeled cells were mounted with an anti-fade medium (Biomeda Gel Mount, Electron Microscopy Sciences, Foster City, CA, USA) and observed under a confocal Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) equipped with a HeNe/Ar laser source for fluorescence measurements. Observations were performed using a Leica Plan Apo 63X/1.43NA oil immersion objective. Series of optical sections (1024 x 1024 pixels each; pixel size 204.3 nm) 0.4 μm in thickness were taken through the depth of the cells at intervals of 0.4 μm. Images were then projected onto a single 'extended focus' image. Densitometric analysis of the intensity of Notch-ICD fluorescent signal was performed on digitized images using Scion Image Beta 4.0.2 image analysis program (Scion Corp) in 20 regions of interest (ROI) of 100 μm^2 for each confocal stacks (at least 10).

Statistical analysis

The reported data are expressed as the mean ± SEM of at least 3 independent experiments. As the experimental values in each group approximated to a normal distribution, statistical comparison of

differences between groups was carried out using one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. A p value ≤ 0.05 was considered significant. Calculations were done using GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA, USA).

Results

RLX receptor is expressed by cardiac muscle cells

Primary mouse cardiomyocytes were previously found to express RXFP1 (Formigli et al., 2009). The present findings show that rat cardiac muscle H9c2 cells also expressed the specific RLX receptor RXFP1, as shown by RT-PCR and Western blotting (Figure 4 A,B).

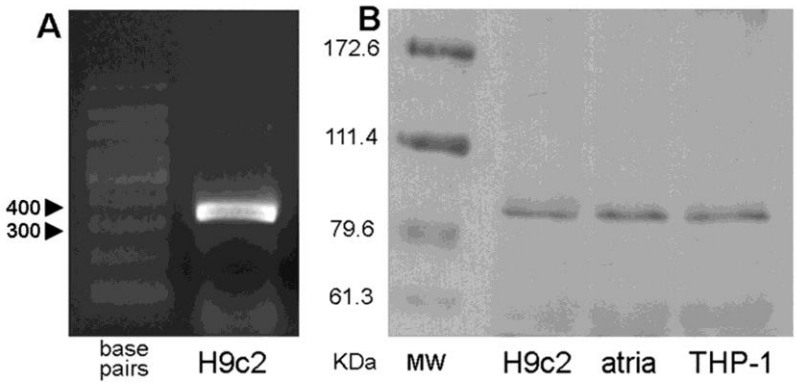

Fig.4 RT-PCR (A) and Western blotting (B) showing RXFP1 RLX receptor expression by H9c2 rat cardiac muscle cells

RLX increases cardiac muscle cell viability impaired by H+R.

The trypan blue exclusion test (Figure 5 A) showed that hypoxia and especially reoxygenation caused a marked reduction of H9c2 cell viability. RLX (17 nmol/l), added to the culture medium 24 h before hypoxia, significantly increased cell viability both after hypoxia and after reoxygenation. RLX also had protective effects when added at reoxygenation. The beneficial effects of RLX were significantly reduced, albeit not abolished, when the Notch-1 inhibitor DAPT was administered together with the hormone, indicating that Notch-1 signaling is involved in the cardioprotective role of RLX against cardiac injury (Figure 5 A).

Similar findings were obtained with the MTT assay (Figure 5 B), which showed that hypoxia and reoxygenation caused a marked reduction of H9c2 cell viability. RLX (17 nmol/l), added 24 h before hypoxia, significantly increased cell viability both after hypoxia and after reoxygenation. RLX also had protective effects when added at reoxygenation. iRLX substituted for authentic RLX resulted in the disappearance of any cytoprotective effect, as evaluated by the MTT assay on H9c2 cells (Figure 5 B).

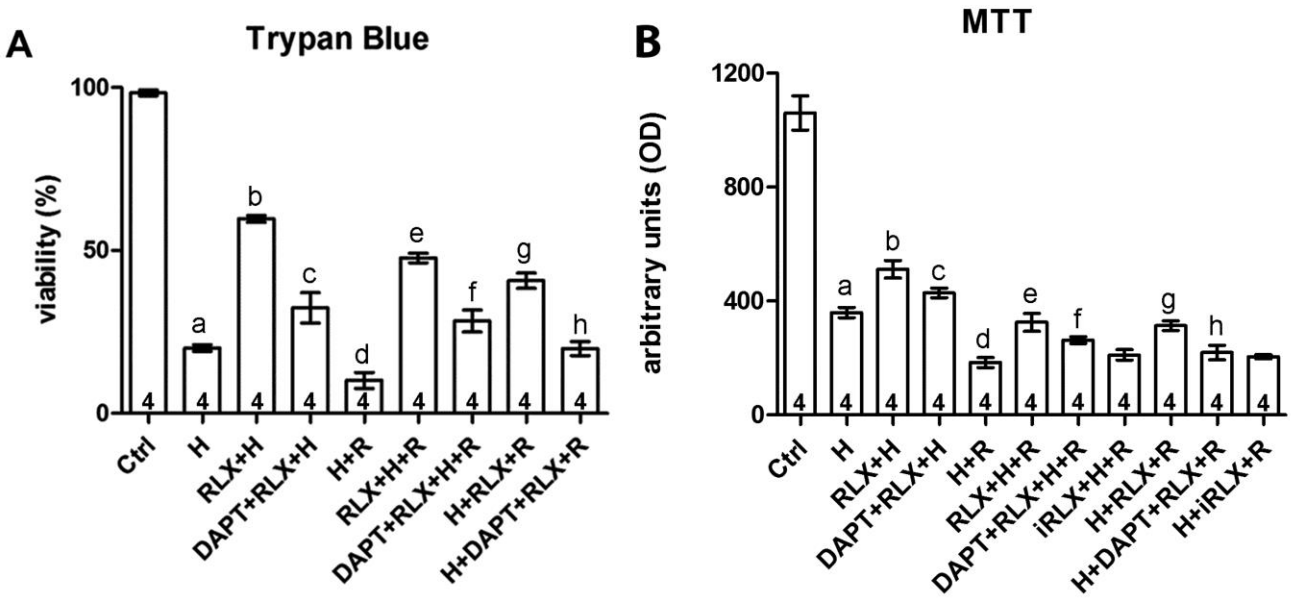

Fig. 5 Evaluation of H9c2 cell viability by trypan blue exclusion (A) and MTT (B) assay. Replicas of the experiments (n) are indicated at the bottom of each column. Values are mean ± SEM. Significance of differences for trypan blue assay: a, $p<0.001$ vs. control; b, $p<0.001$ vs. H; c, $p<0.001$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.05$ vs. H; e, $p<0.001$ vs. H+R; f, $p<0.001$ vs. RLX+H+R; g, $p<0.001$ vs. H+R; h, $p<0.001$ vs H+RLX+R. Significance of differences for MTT assay: a, $p<0.001$ vs. control; b, $p<0.01$ vs. H; c, $p<0.05$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.01$ vs. H; e, $p<0.01$ vs. H+R; f, $p<0.05$ vs. RLX+H+R; g, $p<0.01$ vs. H+R; h, $p<0.05$ vs H+RLX+R .

Light microscopic observation of H9c2 cell monolayers confirmed the above findings (Figure 6): in fact, the cell amounts were markedly reduced and the individual cell morphology worsened by hypoxia and H+R, whereas RLX reversed the adverse effects. Co-administration of DAPT reduced the effects of RLX.

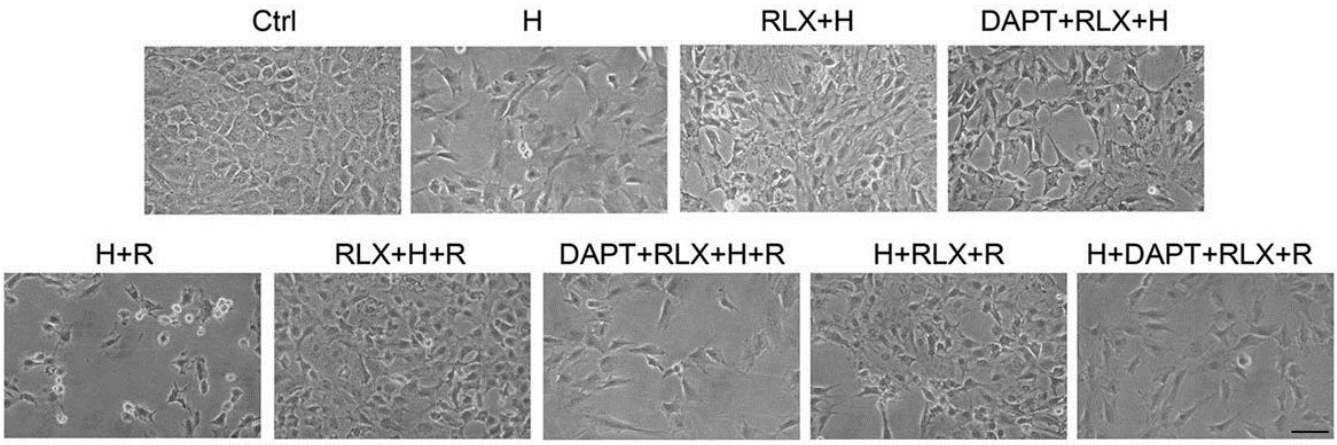

Fig. 6 Representative phase contrast micrographs of H9c2 cell monolayers. Scale bars 20 μm.

The cardioprotective action of RLX against hypoxia- and H+R-induced cell death was confirmed in parallel experiments with primary cultures of mouse neonatal cardiomyocytes subjected to trypan blue exclusion and MTT assays, which gave similar results as those performed on H9c2 cells (Figure 7 A,B).

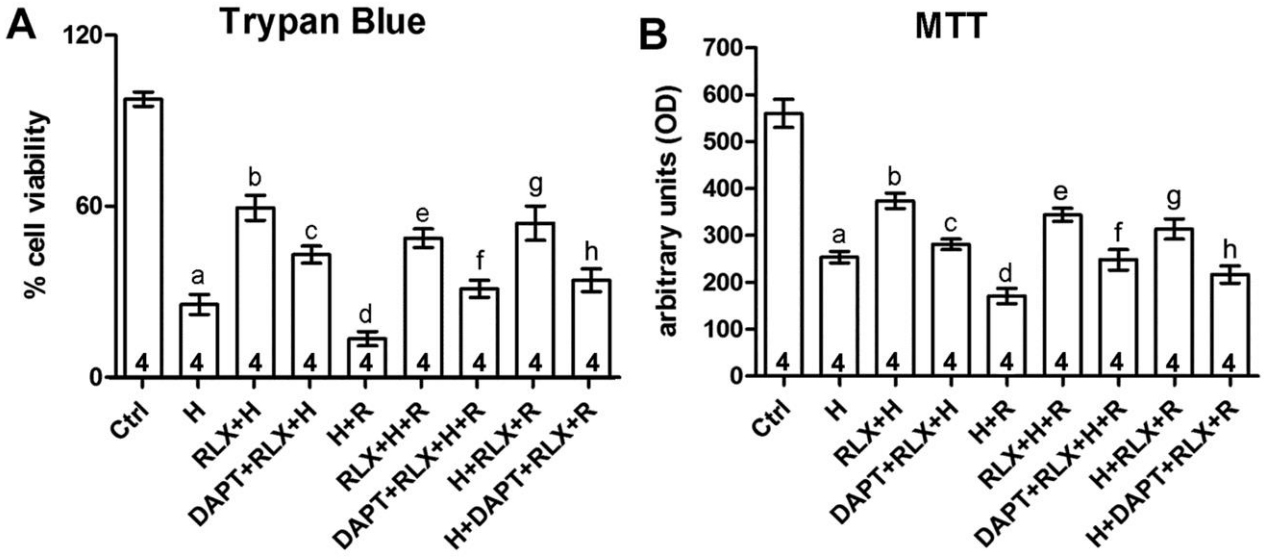

Fig.7 Evaluation of the viability of mouse cardiomyocytes in primary culture by trypan blue exclusion (A) and MTT (B) assay. Replicas of the experiments (n) are indicated at the bottom of each column. Values are mean ± SEM. Significance of differences for trypan blue assay: a, $p<0.001$ vs. control; b, $p<0.001$ vs. H; c, $p<0.01$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.05$ vs. H; e, $p<0.001$ vs. H+R; f, $p<0.01$ vs. RLX+H+R; g, $p<0.001$ vs. H+R; h, $p<0.05$ vs H+RLX+R . Significance of differences for MTT assay: a, $p<0.001$ vs. control; b, $p<0.01$ vs. H; c, $p<0.01$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.01$ vs. H; e, $p<0.001$ vs. H+R; f, $p<0.01$ vs. RLX+H+R; g, $p<0.01$ vs. H+R; h, $p<0.05$ vs H+RLX+R .

RLX protects cardiac muscle cells from nitroxidative damage induced by H+R.

RLX increased H9c2 cell viability by reducing nitroxidative stress occurring at reoxygenation (Figure 8). In fact, the levels of immunoreactive nitrotyrosine, a marker of protein nitration which were enhanced upon H+R, were significantly reduced after the addition of RLX, either before hypoxia or, at a lesser extent, at reoxygenation (Figure 8 A). Similar findings were observed in the experiments performed to quantify oxidized DNA. The levels of 8-OHdG were enhanced upon H+R, were significantly reduced after the addition of RLX, either before hypoxia or, at a lesser

extent, at reoxygenation) (Figure 8 B). Of interest, the protective effects of RLX against oxidative stress were reduced by DAPT co-administration (Figure 8 A,B).

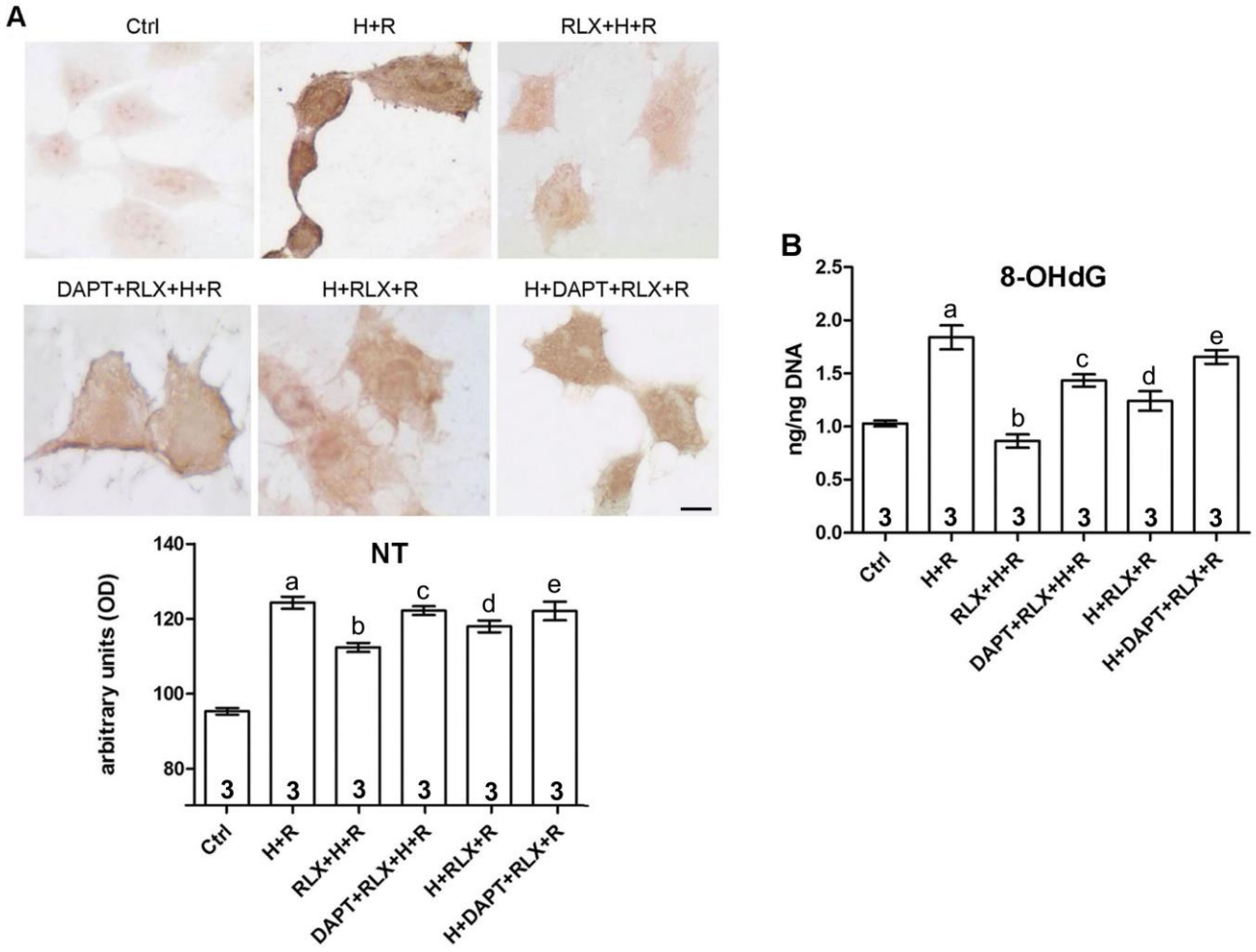

Fig.8 Evaluation of H9c2 cell nitroxidative stress by immunoreactive nitrotyrosine (A) and 8-OHdG (B). Scale bars 10 μm. Replicas of the experiments (n) are indicated at the bottom of each column. Values are mean ± SEM. Significance of differences for nitrotyrosine: a, $p<0.001$ vs. control; b, $p<0.001$ vs. H+R; c, $p<0.001$ vs. RLX+H+R; d, $p<0.05$ vs. H+R; e, $p<0.05$ vs. H+RLX+R. Significance of differences for 8-OHdG: a, $p<0.01$ vs. control; b, $p<0.001$ vs. H+R; c, $p<0.01$ vs. RLX+H+R; d, $p<0.01$ vs. H+R; e, $p<0.05$ vs. H+RLX+R.

RLX protects cardiac muscle cells from apoptosis induced by H+R.

RLX significantly decreased apoptotic death induced by hypoxia and reoxygenation in H9c2 cells. Indeed, compared with the controls, mRNA expression of the antiapoptotic gene bcl2 was reduced and that of the proapoptotic gene bax enhanced by hypoxia and, even more, by reoxygenation (Figure 9). RLX, given both before ischemia and at reperfusion, increased the expression of bcl2 and decreased that of bax. The effects of RLX were reduced by co-administration of DAPT (Figure 9). Evaluation of the percentage of TUNEL-positive apoptotic cells was consistent with these findings (Figure 10). Hypoxia and especially reoxygenation caused a marked increase of apoptosis. RLX (17 nmol/l), added to the culture medium 24 h before hypoxia, significantly decreased apoptosis both after hypoxia and after reoxygenation. RLX also had protective effects when added at reoxygenation. As expected, co-administration of DAPT reduced the effects of RLX (Figures 9,10). RLX also significantly decreased apoptotic cell death induced by hypoxia and reoxygenation in H9c2. Indeed, the mRNA expression of the antiapoptotic gene bcl2 was reduced and that of the proapoptotic gene bax enhanced by hypoxia and, even more, by reoxygenation (Figure 9). Consistently, the percentage of TUNEL-positive apoptotic cells was increased upon hypoxia and reoxygenation (Figure 10). Treatment with RLX before hypoxia or at reoxygenation (Figures 9,10) significantly attenuated these changes. As expected, co-administration of DAPT reduced the effects of RLX (Figures 9, 10).

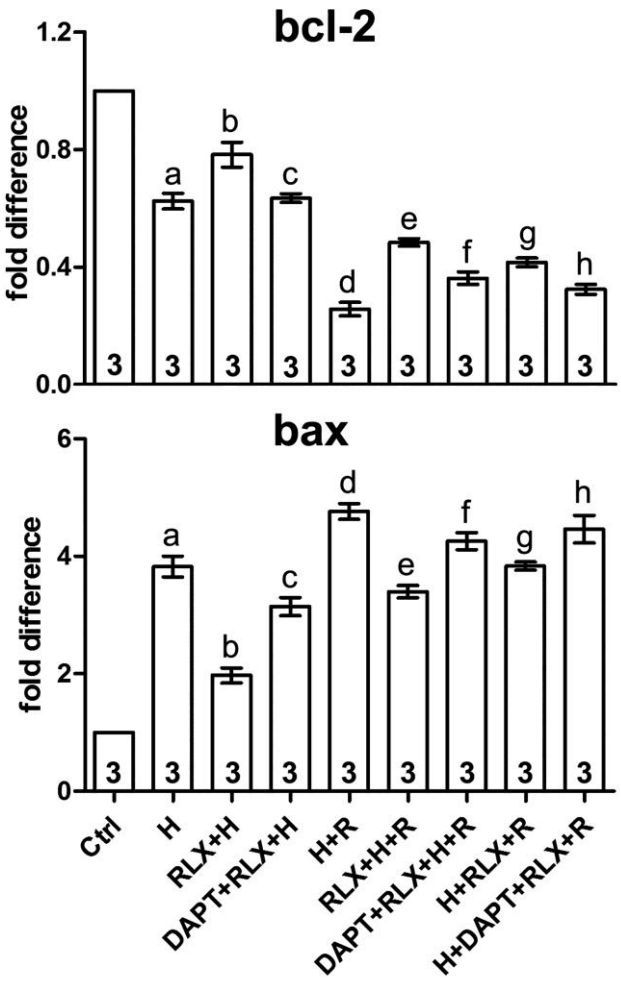

Fig.9 Evaluation of the expression of the antiapoptotic gene *bcl2* and of the proapoptotic gene *bax* in H9c2 cells by real-time PCR. Replicas of the experiments (n) are indicated at the bottom of each column. Values are mean ± SEM. Significance of differences for bcl-2: a, $p<0.001$ vs. control; b, $p<0.01$ vs. H; c, $p<0.01$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.001$ vs. H; e, $p<0.001$ vs. H+R; f, $p<0.01$ vs. RLX+H+R; g, $p<0.01$ vs. H+R; h, $p<0.05$ vs H+RLX+R . Significance of differences for bax: a, $p<0.001$ vs. control; b, $p<0.001$ vs. H; c, $p<0.001$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.05$ vs. H; e, $p<0.001$ vs. H+R; f, $p<0.01$ vs. RLX+H+R; g, $p<0.01$ vs. H+R; h, $p<0.05$ vs H+RLX+R.

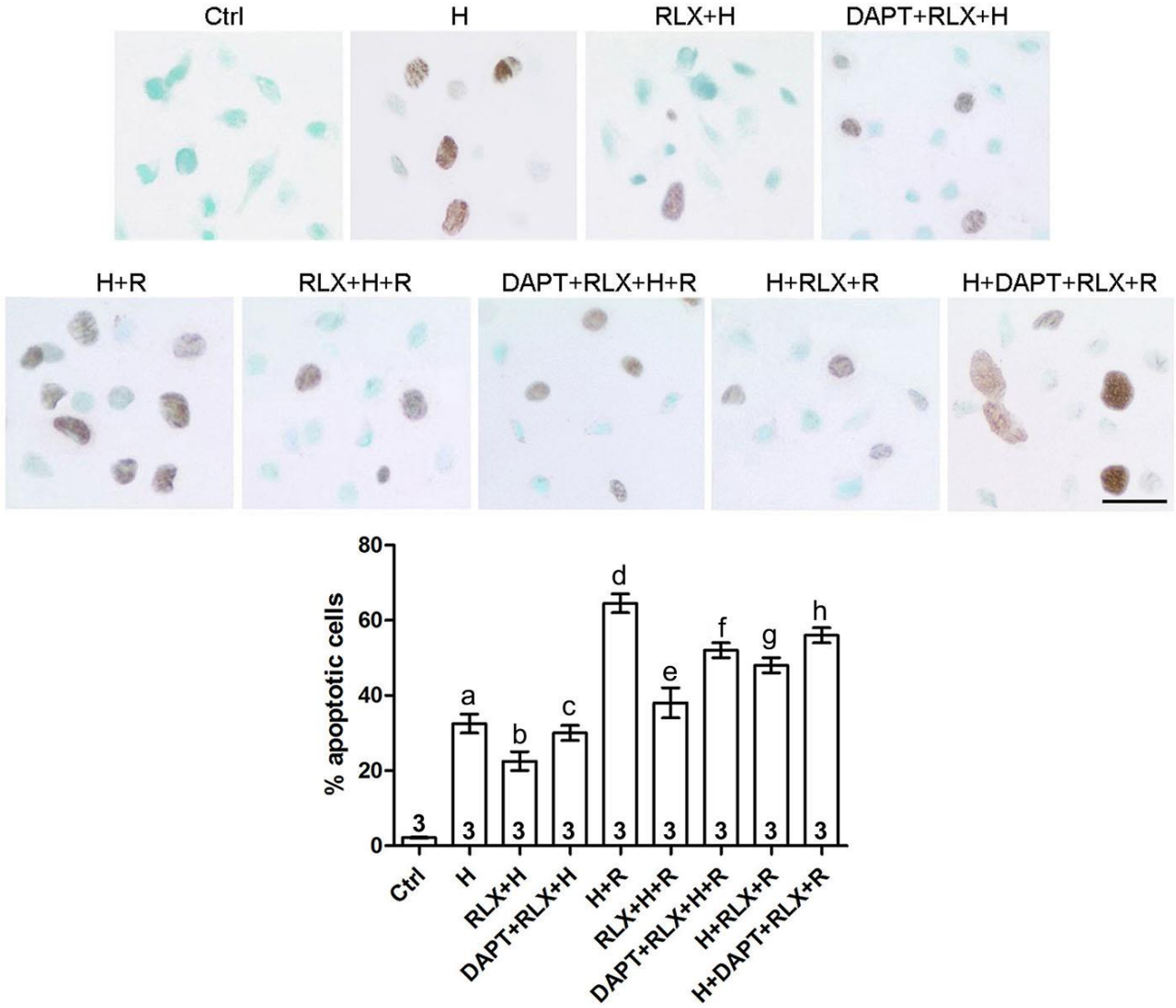

Fig.10 Evaluation of apoptotic H9c2 cells by TUNEL assay. Scale bars 10 μm. Replicas of the experiments (n) are indicated at the bottom of each column. Values are mean ± SEM. Significance of differences: a, $p<0.001$ vs. control; b, $p<0.05$ vs. H; c, $p<0.05$ vs. H+RLX; d, $p<0.001$ vs. control and $p<0.001$ vs. H; e, $p<0.001$ vs. H+R; f, $p<0.01$ vs. RLX+H+R; g, $p<0.01$ vs. H+R; h, $p<0.05$ vs H+RLX+R.

Transmission electron microscopy confirmed the cardioprotective effects of RLX (Figure 11). Indeed, H9c2 cells subjected to hypoxia and H+R showed signs of damage typical of apoptosis, namely cytoplasmic shrinkage and vacuolation, mitochondrial swelling and chromatin condensation or fading. These morphologic features were, instead, rarely observed in the RLX-treated cells. Co-administration of DAPT and RLX resulted in prominent signs of cell damage.

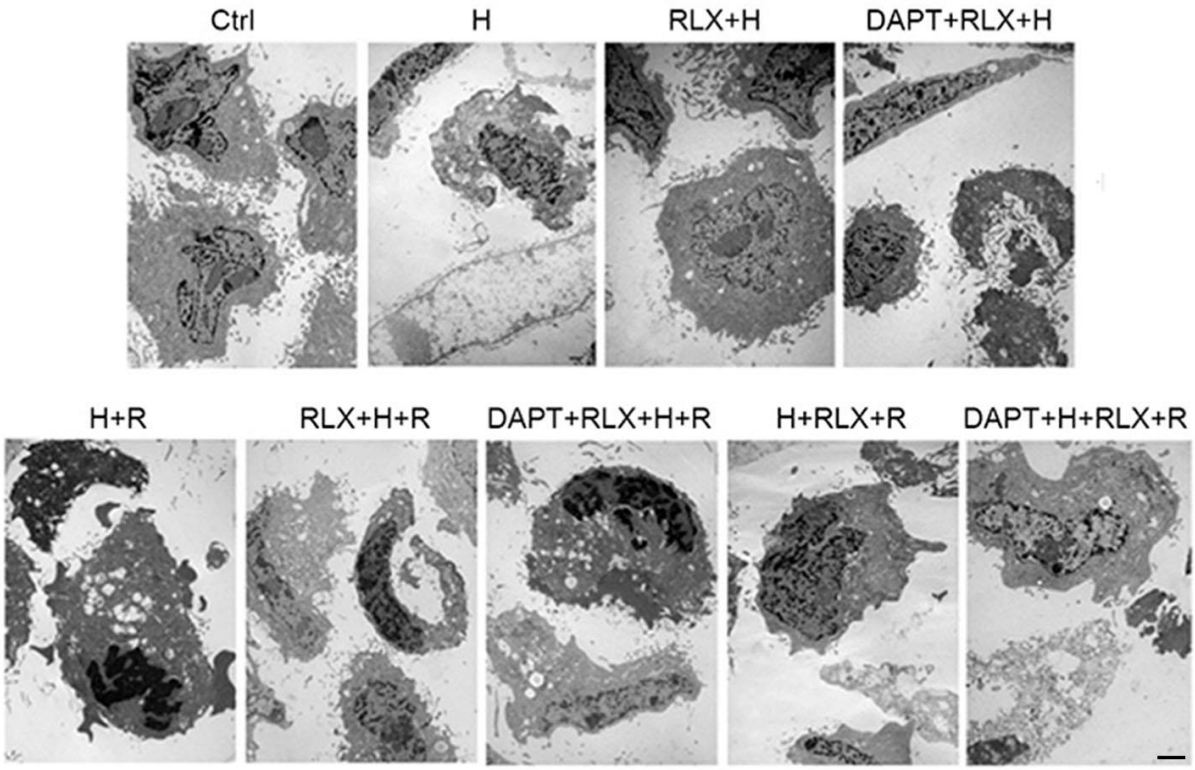

Fig.11 Representative transmission electron micrographs of H9c2 cells in the different experimental conditions. Scale bar 1μm.

RLX stimulates Notch-1 signalling pathway

The above findings indicate that inhibition of Notch-1 signaling pathway reduces the cardioprotective effects of RLX, suggesting a close functional relationship. Indeed, a 24-h treatment of H9c2 cells with RLX (17 nmol/l) induced a significant increase in Notch-ICD expression, the active intracellular fragment of Notch-1 receptor (Figure 12 A,B). As expected, DAPT significantly reduced the basal expression of Notch-ICD (Figure 12 A,B).

Of note, in the current experimental conditions, Notch-ICD was down-regulated by hypoxia and, even more, by reoxygenation (Figure 12 C,D), whereas RLX, given either 24 h before hypoxia or at reoxygenation, antagonized such effect (Figure 12 C,D).

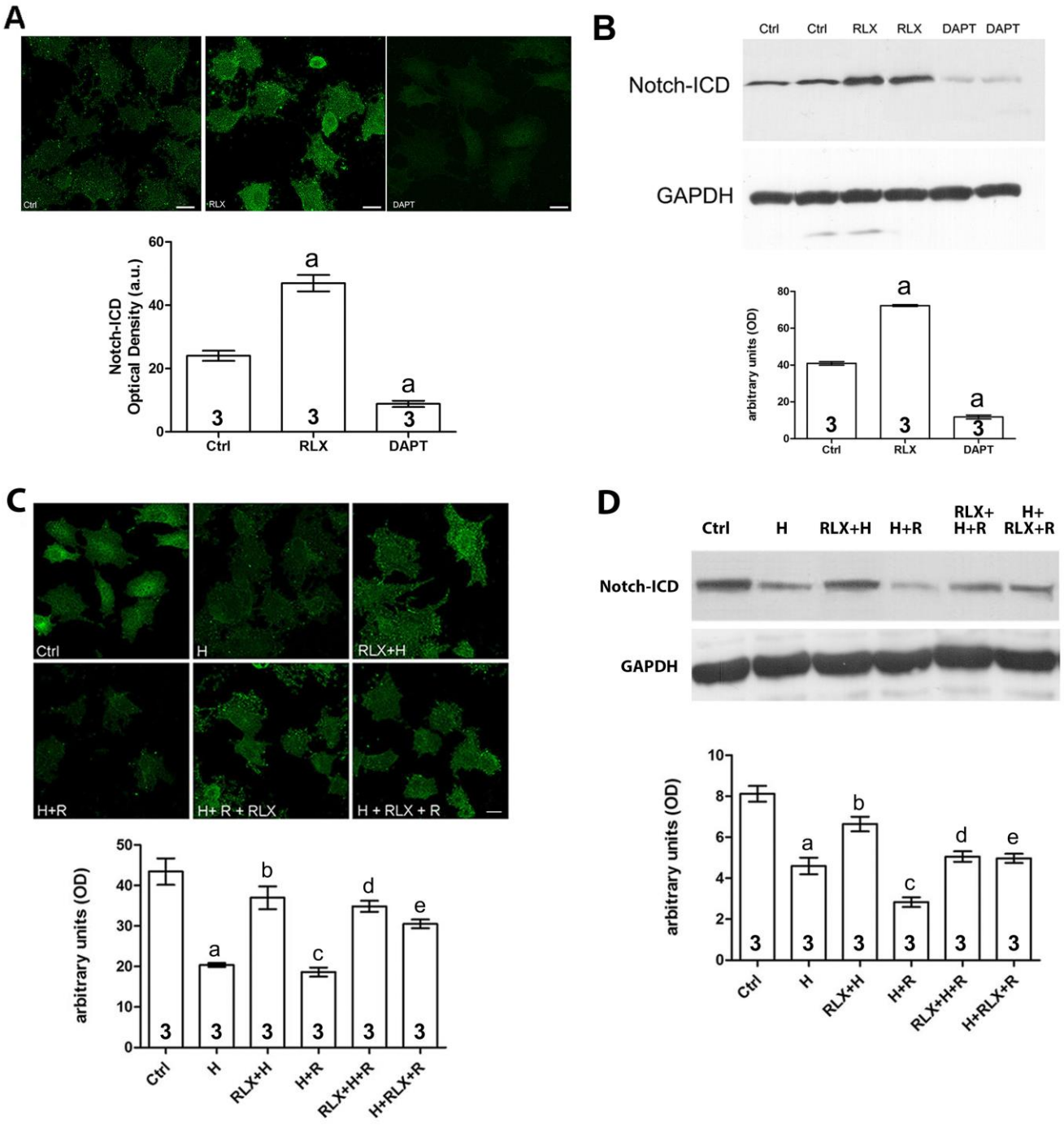

Fig.12 Evalution of Notch-1 activation by RLX in H9c2 cells under the different experimental conditions by confocal immunofluorescence (A,C) and Western blotting (B,D). Scale bar 10 μm. Replicas of the experiments (n) are indicated at the bottom of each column. Values are mean ± SEM. Significance of differences: panels A,B): a, $p<0.001$ vs. control; panel C): a, $p<0.001$ vs. control; b, $p<0.001$ vs. H; c, $p<0.001$ vs. control and $p<0.05$ vs. H; d, $p<0.001$ vs. H+R; e, $p<0.05$ vs. H+R; panel D): a, $p<0.01$ vs. control; b, $p<0.05$ vs. H; c, $p<0.001$ vs. control and $p<0.01$ vs. H; d, $p<0.05$ vs. H+R; e, $p<0.01$ vs. H+R.

Discussion

Identification of the endogenous mechanisms that the heart put into play to increase its resistance to adverse conditions represents a new frontier of cardiologic research. In this context, agents capable of modulating these mechanisms to improve myocardial rescue upon an ischemic insult can be a promising therapeutic approach (Della-Morte et al., 2012). Among such agents, RLX deserves a pivotal place. Convincing evidence exists in the literature that RLX, which has been credited as a cardiotropic hormone (Bani 1997; Dschietzig and Stangl, 2002; Nistri et al., 2007) protects the heart from ischemia and reperfusion-induced myocardial damage in *ex vivo* and *in vivo* models through its vasodilator, anti-inflammatory and anti-fibrotic actions (Bonacchi et al. 2009; Nistri et al., 2003; Masini et al., 2004; Perna et al., 2005; Samuel et al., 2004; Samuel et al., 2011; Lekgabe et al., 2005; Formigli et al., 2007; Sassoli et al., 2013). The current findings provide first information that RLX also has a direct cytoprotective effect on cardiac muscle cells subjected *in vitro* to hypoxia and reoxygenation increasing their resistance to oxygen deprivation and nitroxidative stress. This finding is in keeping with the observation that RLX protects cardiomyocytes against oxidative stress-induced apoptosis (Moore et al., 2007). The RLX-induced protection was observed both in H9c2 cells, a cardiac muscle lineage currently used as a model for ischemic injury, and in primary cultures of freshly isolated ventricular cardiomyocytes. In particular, our findings demonstrate that the protective action of RLX is receptor-specific, because it is completely abolished by iRLX, and involves the activation of Notch-1 signaling pathway. Notch-1 is implicated in cardiac development, where it regulates cardiomyocyte proliferation, myocardial trabeculation and valve formation (Niessen and Karsan, 2007; High and Epstein 2008) as well as in the maintenance of adult heart tissue integrity (Campa et al., 2008; . Collesi et al., 2008; Nemir and Pedrazzini, 2008). Notch signaling also plays a role in heart disease: its expression increases in myocardial infarction (Gude et al., 2008; Kratsios et al., 2010) and in dilated or hypertrophic cardiomyopathy (Croquelois et al., 2008). Notch-1 activation contributes to cardioprotection afforded by ischemic pre- and postconditioning and in the reduction of ischemia/reperfusion-induced cardiac nitroxidative stress in *in vitro* and *in vivo* animal models (Yu and Song, 2013; Zhou et al., 2013; Pei et al., 2013). The present findings indicate that H9c2 cardiac muscle cells, which express the high affinity RLX receptor RXFP1, respond to RLX by up-regulating the expression of Notch-ICD, the active intracellular domain of Notch-1. Indeed, Notch-1 activation requires its proteolytic cleavage by ADAM metalloproteases and γ-secretase, causing the release of Notch-ICD, which translocates into the nucleus and stimulates the transcription of target genes controlling cell proliferation and maturation (Boni et al., 2008). In the present experimental conditions, Notch-ICD expression was

markedly decreased in hypoxic and H+R-exposed H9c2 cells. RLX, added either 24 h before hypoxia or at reoxygenation, was capable of significantly reducing the Notch-ICD down-regulation. This mechanism plays a major role in the cytoprotective action of RLX on cardiac muscle cells. In fact, the capability of RLX to increase cell viability by reducing H+R-dependent oxidative stress and apoptosis is significantly hampered by co-administration of DAPT, a γ-secretase inhibitor that blocks the generation of Notch-ICD (Brou, 2009). The fact that DAPT does not completely abolish the effects of RLX may suggest that multiple cytoprotective signaling pathways are operated by RLX in cardiac muscle cells. For instance, these pathways may involve the endogenous generation of nitric oxide, a typical downstream effector of RLX (Baccari and Bani, 2008), which has been demonstrated to protect cardiomyocytes from H+R-induced apoptosis (Rakhit, et al., 2001). The possible mechanisms by which RLX can activate the Notch-1 pathway remain to be elucidated. It can be speculated that RLX may induce ADAM 10 activation through a dual pathway which involves the up-regulation of Akt, a downstream pathway of RXFP1 activation (McGuane et al., 2011) also involved in ADAM 10 activation (Fernandez et al., 2010), and down-regulation of TIMP-1 (Palejwala et al., 2001), a known ADAM 10 inhibitor (Edwards et al., 2008).

We shall point out that the present findings have been obtained on cultured embryonic and neonatal cardiac muscle cells, which may not exactly behave as adult cardiomyocytes of the heart *in vivo*. On the other hand, this fact may suggests that RLX is capable of preserving viability of the scattered population of cardiac stem cells of the adult heart, favoring their post-ischemic activation as an attempt to replace damaged cardiomyocytes. In this context, the finding that RLX constitutively activates the Notch-1 pathway in H9c2 cardiac muscle cells may contribute to explain the mechanisms whereby RLX promote neonatal cardiomyocyte growth and maturation (Nistri et al., 2012). In fact, previous reports have shown that Notch-1 induces cell cycle re-entry and proliferation of cardiac muscle cells (Campa et al., 2008; Collesi et al., 2008). However, whether RLX may also be cardioprotective through the recruitment of cardiac muscle precursors in the post-infarcted heart remains a stimulating matter for further investigation.

Our findings indicate that the cytoprotection afforded by RLX is more potent when the hormone was added before the occurrence of hypoxia than at the onset of reoxygenation. This notion, taken together with the previous reports that cardiomyocytes produce RLX (Taylor and Clark, 1994) and express specific RLX receptors (Kompa et al., 2002; Moore et al., 2014), strongly suggests that RLX could be an endogenous cardiac factor involved in the mechanisms of myocardial preconditioning. Further clues in support to this hypothesis come from the observations that: i) plasma RLX is increased in patients with cardiac failure (Dschietzig et al., 2001) suggesting that RLX may be released from the injured heart as an attempt to compensate for noxious conditions; ii)

Notch-1 signaling has been recognized as a major endogenous mechanism involved in heart pre- and postconditioning (Yu and Song, 2013; Zhou et al., 2013). Nonetheless, RLX also exerted a significant cytoprotective action when administered at reoxygenation, suggesting that it can effectively counteract the acute cellular mechanisms of reperfusion injury.

In conclusion, our study expands the knowledge of the pharmacological properties of RLX as a cardioprotective agent. Of note, phase III clinical trials performed on heart failure patients have demonstrated that human recombinant RLX, or serelaxin (Novartis, Basel, Switzerland), significantly improved the hemodynamic parameters and life expectancy of the treated patients compared to the placebo group (Teerlink et al., 2013) thus emerging as a new drug for the treatment of the failing heart. The existing experimental background can justify the design of clinical trials to explore whether RLX may be a new drug for the primary and secondary prevention and therapy of ischemic heart disease.

$Mn^{II}(Me_2DO2A)$

Mn^{II}(4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetate), also termed $Mn^{II}(Me_2DO2A)$, is a non-peptidic, low molecular weight, Mn -containing compound, capable to catalyze $O_2^{\bullet-}$ dismutation. This molecule has been designed, synthesized and characterized, chemically and biologically, by a multi-disciplinary research team of the University of Florence, involving researchers from the Departments of Chemistry, NEUROFARBA, and Experimental & Clinical Medicine. The compound has been patented and is now property of General Project Ltd. (Montespertoli, Florence, Italy), which is gratefully acknowledged for kindly giving the permission to use this compound for the present studies.

Role of reactive oxygen species (ROS) in I/R injury

Multiple interplaying mechanisms play a role in I/R damage, mainly consisting in production of reactive oxygen species (ROS) (Jaeschke and Woolbright, 2012). Pathogenic ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hydroxyl radical ($HO^{\bullet}$) and perhydroxyl radical (HO_2). All of them are formed by $O_2^{\bullet-}$ reduction reactions occurring in the cellular microenvironment. Among ROS, $O_2^{\bullet-}$ is the main representative and widely investigated for its pathological relevance. In normal conditions, the endogenous levels of $O_2^{\bullet-}$ are controlled by superoxide dismutases (SOD), located in mitochondria (Mn-SOD), cytosol (Cu-/Zn-SOD), and outer plasma membrane (Cu- /Zn-SOD). SOD catalyze the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 by a reaction requiring a key transition metal at the catalytic site (Fridovich 1989; Johnson and Giulivi, 2005). The resultant H_2O_2 is then degraded to O_2 and H_2O by cytosolic or mitochondrial catalase, glutathione peroxidase (Thannickal and Fanburg, 2000) and peroxiredoxin (Vivancos et al., 2005). On the other hand, whenever the generation of $O_2^{\bullet-}$ is enhanced over the inactivating capability of SODs, $O_2^{\bullet-}$-mediated cell injury occurs (Finkel, 2005). Moreover, oxidative stress causes a rapid inactivation of SODs, sparkling a vicious cycle that causes ever-increasing, harmful $O_2^{\bullet-}$ tissue levels (Wang et al., 2004).

In particular, mitochondrial dysfunction of hypoxia-exposed cells impairs the electron flow and enhance $O_2^{\bullet-}$ formation and, therefore, release of ROS (Murphy 2009; Poyton et al., 2009). The over-production of ROS during I/R determines a depolarization of mitochondrial membrane

potentials (Crompton, 1999; Levraut et al., 2003), an increase of mitochondrial permeability transition pores (MPTP) and of intracellular calcium concentration (Crompton, 1999; Levraut et al., 2003; Li and Jackson, 2002). All these events determine cell damage and, finally, cell death. Moreover, mitochondria can use the respiratory chain to reduce nitrite (NO_2^-) to nitric oxide ($NO^\bullet$) (Castello et al., 2008). Because cytochrome oxidase produces $NO^\bullet$ from nitrite at low O_2 concentrations, the mitochondrially generated oxidants whose concentration increase under hypoxic conditions also include peroxynitrite ($ONOO^-$), which is formed by the reaction between $O_2^{\bullet-}$ and $NO^\bullet$ (Galkin et al., 2007). ONOO- is a relatively long-lived, stable molecule, which has the ability to reach critical targets of cells, as it is membrane permeable (Glebska and Koppenol, 2003); it is involved in myocardial cytotoxicity by direct oxidation of lipids, proteins, and DNA (Pesse et al., 2005; Pacher et al., 2005) . Over the past decade, $ONOO^-$ has been identified as the major harmful oxidant in myocardial hypoxia-reoxygenation injury (Xie et al., 1998).

Inflammatory cells recruited upon endothelial injury also provide a major contribution to oxidative stress through the production of both $O_2^{\bullet-}$ and $NO^\bullet$, and hence, $ONOO^-$.

The above notions underscore that $O_2^{\bullet-}$ and $NO^\bullet$ play a pivotal role in oxidative stress upon hypoxia-reoxygenation and hence can be suitable target of new pharmacological interventions.

Non-peptidic, low molecular weight, metal-containing compounds as ROS scavengers

At first, pharmacological research was based on the use of extractive or recombinant SOD as therapeutic approach to oxidative stress upon I/R, in order to allow removal of excess $O_2^{\bullet-}$ and leaving enough $NO^\bullet$ to sustain residual vascular function (Seal and Gewertz, 2005). Indeed, transfection of H9c2 rat cardiomyocytes with Cu/Zn SOD was found to attenuate I/R injury (Liu et al., 2013). However, the pharmaceutical use of SOD is hampered by major issues, including poor stability in water, limited intracellular penetration, immunogenicity, short half-life, and unfavorable yield/cost ratio (McCord and Edeas, 2005; Slemmer et al., 2008). Pharmacological research was then oriented toward of non-peptidic, low molecular weight, metal-containing compounds capable to efficiently catalyze $O_2^{\bullet-}$ dismutation (Riley 1999). These complexes are based on a well-known principle of inorganic chemistry, i.e. the property of transition metals to bind to and react with free radicals. In the last three decades, a number of manganese, iron, copper and nickel complexes, have

been used as SOD-mimetic agents (Riley 1999; Zhang and Lippard, 2003; Cuzzocrea et al., 2004; Riley and Schall, 2006; Batinic-Haberle et al., 2011). Among them, manganese and iron complexes were generally found to be the most effective. However, the iron complexes often resulted to be toxic *in vivo* (Riley 1999; Zhang and Lippard, 2003; Cuzzocrea et al., 2004; Riley and Schall, 2006; Batinic-Haberle et al., 2011; Batinić-Haberle et al., 1999). Therefore, in the last decade, major attention has been paid to manganese complexes. Basically, four different classes of Mn-based compounds have been synthesized, namely Mn^{II} complexes with pentaazamacrocycles, polyamine-polycarboxylates, salen derivatives and porphyrins (Muscoli 2003; Batinic-Haberle et al. 2011; Baker et al. 1998; Salvemini 1999; Salvemini et al., 2002; Sheng 2002; Batinic-Haberle 2010; Miriyala et al., 2012). They have been tested as ROS scanvangers for therapeutic purposes in cellular and animal models of oxidative stress (Bani and Bencini, 2012).

$Mn^{II}(Me_2DO2A)$

In recent years, a series of studies have been carried out on a polyamine-polycarboxylate Mn^{II} complex, Mn^{II}(4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetate), also termed $Mn^{II}(Me_2DO2A)$, patented as $O_2^{\bullet-}$ scavenger.

Polyamine-polycarboxylate scaffolds represent an optimal tool for the synthesis of highly stable Mn^{II} complexes. In fact, these molecules are known for their ability to strongly bind a number of metals, from alkali and alkali earths to lanthanide and transition metal cations, including Mn^{II}. Moreover, these compounds are resistant to oxidizing and reducing agents, are highly soluble in aqueous media, and exhibit low toxicity, all properties that render them suitable for several biological and medicinal applications (Bianchi et al., 2000; Merbach and Tóth, 2001).

Among these, $Mn^{II}(Me_2DO2A)$ presents an organic scaffold with a tetra-amine macrocyclic moiety, characterized by high thermodynamic stability, kinetic inertness and poor tendency to bind alkali or alkaline metal cations (Lindoy 1989). It also possesses two carboxylic moieties as pendant arms, acting as additional binding sites for metal cations. The carboxylic groups deprotonate upon Mn^{II} binding in aqueous solution, yielding a $Mn^{II}(Me_2DO2A)$ neutral complex, (Figure 13) (Failli et al., 2009).

The stability of $Mn^{II}(Me_2DO2A)$ is also higher than that of other Mn^{II} complexes with endogenous ligands present in the cellular environment, such as ATP , glutathione and carboxylate anions, thus excluding substantial de-metallation of $Mn^{II}(Me_2DO2A)$ by biological chelators.

At the same time, the present ligand yields a Mn^{II} complex remarkably more stable than those with the most abundant metal cations present in intracellular environment, in particular Na^{I}, Mg^{II} and Ca^{II}, and it is unable to bind K^{I}. From an electrochemical point of view, $Mn^{II}(Me_2DO2A)$ displayed only a mono-electronic single oxidation step, related to a $Mn^{II} \rightarrow Mn^{III}$ process, followed by slow rearrangement of the carboxylate groups around Mn^{III}.

Interestingly, the presence of two anionic carboxylate groups neutralizing the charge of Mn^{II} and two lipophilic methyl groups renders $Mn^{II}(Me_2DO2A)$ capable to cross the cell membranes and attain significant intracellular concentrations (Failli et al., 2009), thus being capable to directly scavenge $O_2^{\bullet -}$ in the cytoplasmic environment. The absence of negative charge and increased lipophilia has been shown to facilitate accumulation of manganese complexes in mitochondria (Batinic-Haberle et al., 2014; Spasojevic et al., 2010).

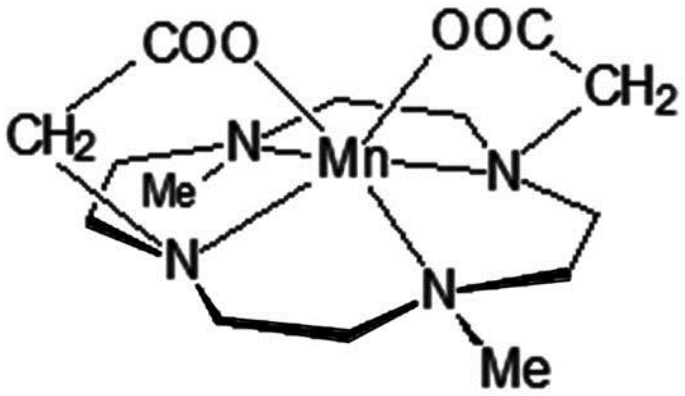

Fig.13 3-D structure of $Mn^{II}(Me_2DO2A)$ (Failli et al., *J Med Chem* 52:7273-7283, 2009)

$Mn^{II}(Me_2DO2A)$, was proven effective in reducing oxidative stress in cell culture models and to decrease oxidative tissue injury in animal models of inflammation (Failli et al., 2009; Cinci et al., 2010; Di Cesare Mannelli et al., 2013). In particular, the $Mn^{II}(Me_2DO2A)$ complex was found to be a potent $O_2^{\bullet -}$ scavenger, provided with biologically relevant effects, in terms of reduced oxidative injury and increased cell viability, in the in the 0.1-1 micromolar range (Failli et al., 2009). It also

had good pharmacokinetic properties, being able to readily cross the plasma membrane and reach effective intracellular concentrations. When given to mice, $Mn^{II}(Me_2DO2A)$ reduced the hyperalgesic response to induced acute and chronic inflammation (Failli et al., 2009); when administered at the same doses to ovalbumin-sensitised guinea pigs, $Mn^{II}(Me_2DO2A)$, it effectively blunted asthma-like reaction, lung inflammation and oxidative injury induced by acute reaction to allergen inhalation (Cinci et al., 2010). $Mn^{II}(Me_2DO2A)$ also behaves as a potent pain reliever in acute inflammatory and chronic articular pain induced in rats. It reduced articular derangement, plasma TNF alpha levels, protein carbonylation and lipid peroxidation (Di Cesare Mannelli et al., 2013).

Aim-2

The aim of the second part of this thesis is to test the antioxidant, cytoprotective potential of $Mn^{II}(Me_2DO2A)$ on cultured H9c2 cardiac muscle cells using an *in vitro* model of I/R – i.e hypoxia followed by reoxygenation (H+R).

Materials and Methods

Reagents

The amount of $Mn^{II}(Me_2DO2A)$ required to perform the present experiments was kindly donated by the patent owner General Project Ltd., Montespertoli (Florence), Italy. The inactive compound $Zn^{II}(Me_2DO2A)$ was synthesized in following the same procedure as $Mn^{II}(Me_2DO2A)$. Unless otherwise specified, the other reagents used for the experiments were from Sigma-Aldrich (Milan, Italy).

Cell culture and treatments

H9c2 embryonic rat cardiac muscle cells, obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 250 U/ml penicillin G and 250 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C. These cells were subjected to hypoxia and reoxygenation (H+R), simulated *in vitro* by substrate starvation plus hypoxia followed by reoxygenation, as previously described. Cells were treated or not with $Mn^{II}(Me_2DO2A)$ at 2 different doses (1 and 10 μmol/l), added at reoxygenation, concurrently with the peak of ROS generation (H+ $Mn^{II}(Me_2DO2A)$+R). The given $Mn^{II}(Me_2DO2A)$ doses were chosen on the basis of previous *in vitro* studies (Failli et al., 2009). As control for the specific capability of $Mn^{II}(Me_2DO2A)$ to suppressing oxidative stress by redox reaction, some cell viability experiments were performed using the inactive congener $Zn^{II}(Me_2DO2A)$ at the same concentrations as $Mn^{II}(Me_2DO2A)$ added at reoxygenation (H+ $Zn^{II}(Me_2DO2A)$+R).

Separate experiments were performed to assess the toxicity of $Mn^{II}(Me_2DO2A)$ and $Zn^{II}(Me_2DO2A)$ in H9c2 cells. In these experiments, the two compounds were added to the cell

cultures at increasing concentrations (0.1-100 μmol/l) for 2 and 24 h; cell viability was then assayed by the MTT test.

Trypan Blue viability assay

The trypan blue exclusion method was used to assess H9c2 cells viability, as previously described.

MTT viability assay

H9c2 mitochondrial function was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described.

Mitochondrial membrane potential (Δψ)

Mitochondrial membrane potential was assessed using tetramethylrhodamine methyl ester perchlorate (TMRM), a lipophilic potentiometric fluorescent dye that distributes between the mitochondria and cytosol in proportion to Δψ by virtue of its positive charge. At low concentrations, the fluorescence intensity depends on dye accumulation in mitochondria, which in turn is directly related to mitochondrial potential. For confocal microscope analysis, cells were cultured on glass coverslips and loaded for 20 min at 37 °C with TMRM, dissolved in 0.1% DMSO to a 100 nM final concentration in the culture medium. The cells were fixed in 2% buffered paraformaldehyde for 10 min at room temperature and the TMRM fluorescence analyzed under a confocal Leica TCS SP5 scanning microscope (Mannheim, Germany) equipped with a helium-neon laser source, using a 543-nm excitation wavelength, and with a Leica Plan Apo x63 oil immersion objective. Mitochondrial membrane potential was also quantified by flow cytometry. Single-cell suspensions were washed twice with PBS and incubated for 20 min at 37 °C in the dark with TMRM dissolved in DMEM (100 nM). The cells were then washed, resuspended in PBS and analyzed using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA).

Mitochondrial permeability transition pore opening (mPTP)

Mitochondrial permeability, an index of mitochondrial dysfunction and early apoptosis, was measured by calcein fluorescence, as described (Petronilli et al., 1999). The fluorescent probe calcein-AM freely enters the cells and emits fluorescence upon de-esterification. Co-loading of cells with cobalt chloride, which cannot cross the mitochondrial membranes in living cells, quenches the fluorescence in the whole cell except mitochondria. During induction of mPTP, cobalt can enter mitochondria and quenches calcein fluorescence, whose decrease can be taken as a measure of the extent of mPTP induction. H9c2 cells grown on glass coverslips were loaded with calcein-AM (3

μM) and cobalt chloride (1 mM) added to the culture medium for 20 min at 37 °C. The cells were then washed in PBS, fixed in 2 % buffered paraformaldehyde for 10 min at room temperature and analyzed by a Leica TCS SP5 confocal laser scanning microscope equipped with an argon laser source, using 488-nm excitation wavelength, and with a Leica Plan Apo x63 oil immersion objective. Mitochondrial permeability was also monitored by flow cytometry: single-cell suspensions were incubated with calcein-AM (3 μM) and cobalt chloride (1 mM) for 20 min at 37 °C, washed twice with PBS and analyzed using a FACSCanto flow cytometer (Becton-Dickinson).

Assessment of Caspase-3 activity

H9c2 cells seeded on glass coverslips were incubated with FAM FLICA™ Caspase 3&7 assay kit (Immunochemistry Technologies, Bloomington, MN, USA) for 30 min, following the manufacturer's instructions. After incubation, the cells were thoroughly washed and fixed in 2% buffered paraformaldehyde for 10 min at room temperature. Fluorescence was detected by a confocal Leica TCS SP5 scanning microscope equipped with an argon laser source, using 488-nm excitation wavelength, and a Leica Plan Apo x63 oil immersion objective. Caspase-3 activity was also quantified by flow cytometry: single-cell suspensions were incubated with FAM-FLICA™ for 30 min at 37 °C, washed twice with PBS and analyzed using a FACSCanto flow cytometer (Becton-Dickinson).

TUNEL assay

Cell death was studied with TUNEL assay, as previously described.

Determination of intracellular ROS and mitochondrial superoxide

H9c2 cells seeded on glass coverslips were loaded with the ROS-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, CA, USA; 2.5 μmol/l) or the mitochondrial $O_2^{\bullet-}$-specific fluorescent probe MitoSOX (Invitrogen; 3 μmol/l) - dissolved in 0.1% DMSO and Pluronic acid F-127 (0.01% w/v) – which were added to cell culture media for 15 min at 37 °C. The cells were fixed in 2% buffered paraformaldehyde for 10 min at room temperature and the H_2DCFDA and MitoSOX fluorescence analysed using a Leica TCS SP5 confocal scanning microscope equipped with an argon laser source, using 488-nm and 543-nm excitation wavelength, respectively, and a Leica Plan Apo x63 oil immersion objective. ROS and mitochondrial $O_2^{\bullet-}$ generation were also monitored by flow cytometry: briefly, single-cell suspensions were incubated with H_2DCFDA (1 μmol/l) or MitoSOX (0.5 μmol/l) for 15 min at 37 °C and immediately analysed

using a FACSCanto flow cytometer (Becton–Dickinson). Data were analyzed using FACSDiva software (Becton–Dickinson).

Immunohistochemical localization and quantitation of nitrotyrosine

Nitrotyrosine (NT), an index of protein nitrosylation, was determined by immunocytochemistry as previously described.

Determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

8-OHdG levels, an indicator of oxidative DNA damage, were determined in H9c2 cells as previously described.

Statistical analysis

The reported data are expressed as the mean ± SEM of at least 3 independent experiments. As the experimental values in each group approximated to a normal distribution, statistical comparison of differences between groups was carried out using one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. A p value ≤ 0.05 was considered significant. Calculations were done using GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA, USA).

Results

$Mn^{II}(Me_2DO2A)$ preserves cardiac muscle cell viability impaired by H+R.

Evaluation of H9c2 cell viability by trypan blue assay (Figure 14) showed that reoxygenation caused a marked reduction of the amounts of viable cells. This effect was inhibited by $Mn^{II}(Me_2DO2A)$ (1 and 10 μmol/l) added at reoxygenation, when high levels of $O_2^{\bullet-}$ are produced. The $Mn^{II}(Me_2DO2A)$-induced cytoprotection showed a dose-dependent trend, as it was expected based on the mechanism of action of $Mn^{II}(Me_2DO2A)$ which involves its functional Mn^{II} centre to scavenge $O_2^{\bullet-}$. Of note, $Zn^{II}(Me_2DO2A)$, made with a similar organic scaffold but lacking Mn^{II}, substituted for $Mn^{II}(Me_2DO2A)$ showed no cytoprotective effect (Figure 14).

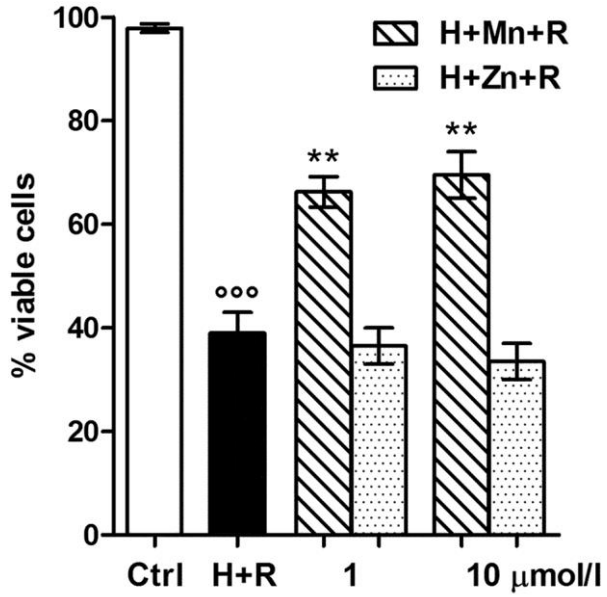

Fig. 14 Evaluation of H9c2 cell viability by trypan blue assay. Significance of differences: °°°p<0.001 vs. control; *p<0.05 and ***p<0.001 vs. H+R.

$Mn^{II}(Me_2DO2A)$ preserves cardiac muscle cell mitochondrial function impaired by H+R.

Parallel experiments to explore mitochondrial integrity and function were carried out with the MTT assay, which reveals the efficiency of the respiratory chain (Figure 15 A) and the TMRM assay, which evaluates the mitochondrial membrane potential (Figure 15 B). The results of these experiments have consistently shown that reoxygenation caused a marked impairment of

mitochondrial function in H9c2 cells. This detrimental effect was significantly blunted by $Mn^{II}(Me_2DO2A)$ (10 μmol/l) added at reoxygenation. Replacement of $Mn^{II}(Me_2DO2A)$ with $Zn^{II}(Me_2DO2A)$ had no protective effect against mitochondrial dysfunction (Figure 15 A,B). FACS analysis confirms the microscopical findings of TMRM fluorescence as it shows that, compared with the control cells, H+R shifts the fluorescence peaks towards higher values (right), while this effect is markedly reduced by $Mn^{II}(Me_2DO2A)$, especially at the higher dose (Figure 15 C).

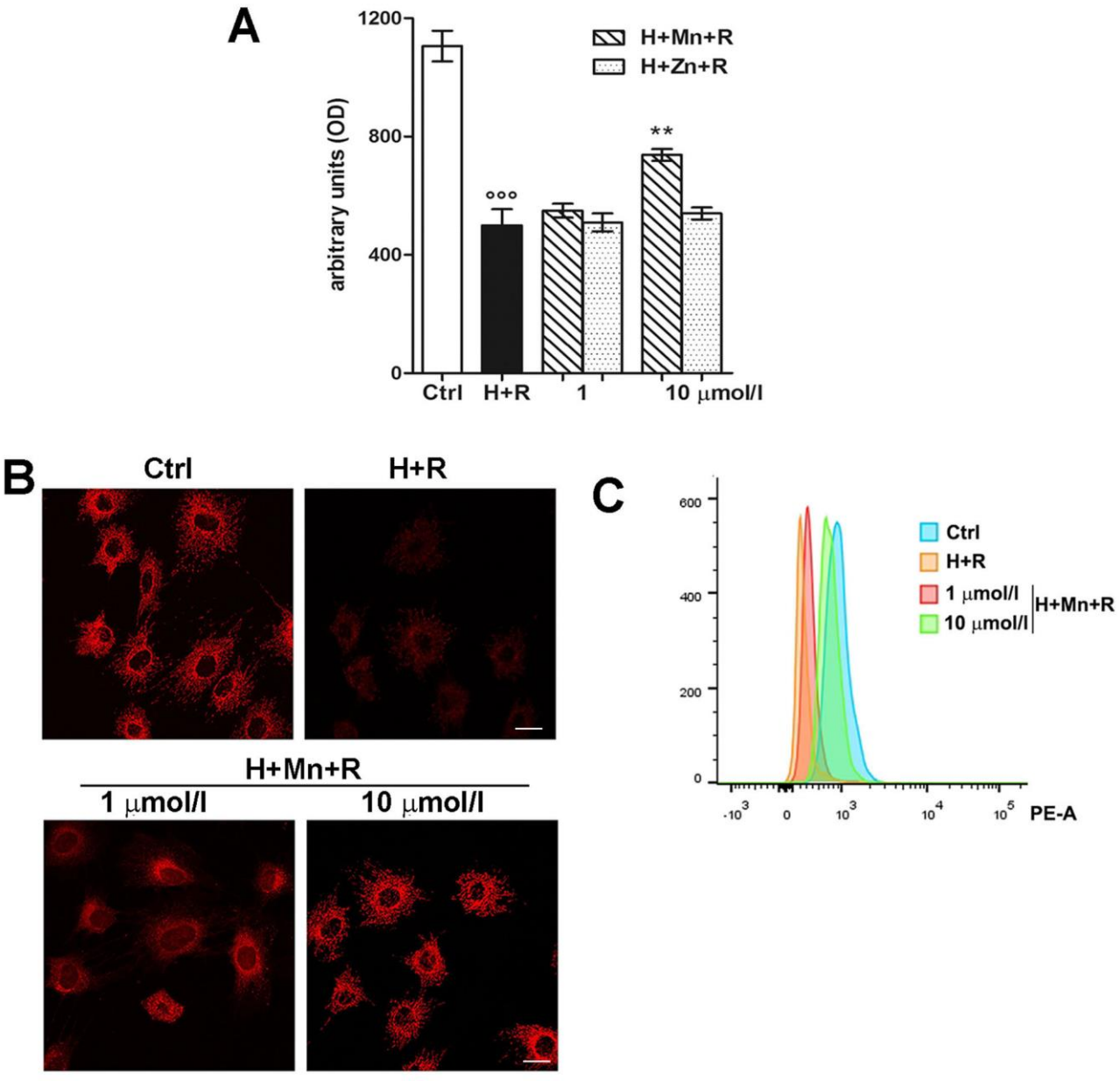

Fig. 15 - Evaluation of H9c2 cell mitochondrial integrity and function by MTT assay (A), TMRM assay by confocal microscopy (B) and FACS analysis (C). Scale bars: 20 μm. Significance of differences: °°°$p<0.001$ vs. control; *$p<0.05$ and ***$p<0.001$ vs. H+R.

$Mn^{II}(Me_2DO2A)$ protects cardiac muscle cells from H+R-induced apoptosis.

The reduction of H+R-induced oxidative stress by $Mn^{II}(Me_2DO2A)$ resulted in a significant decrease in apoptotic cell death. Indeed, the occurrence of mitochondrial permeability transition pores (mPTP) typical of early apoptosis (Figure 16 A,B), and the activation of caspase-3 (Figure 16 A,B) were markedly increased in H9c2 cells exposed to H+R, while the addition of 1 and 10 μmol/l $Mn^{II}(Me_2DO2A)$ at reoxygenation significantly attenuated these changes. FACS analysis confirms the microscopical findings as it shows that, compared with the control cells, H+R shifts the fluorescence peaks towards higher values (right), while this effect is markedly reduced by $Mn^{II}(Me_2DO2A)$, especially at the higher dose (Figure 16 A,B)

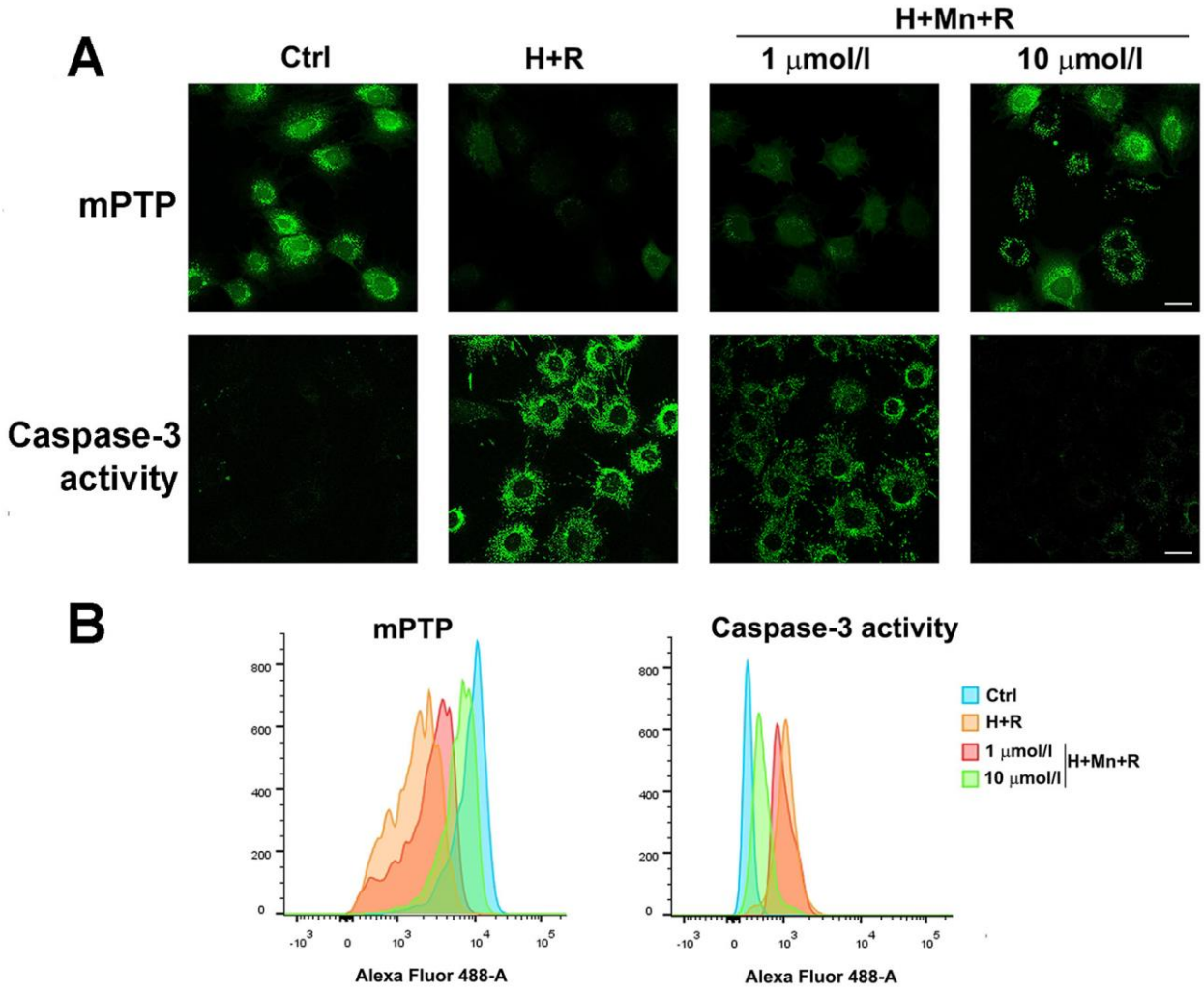

Fig. 16 Evaluation of apoptosis in H9c2 cells by mPTP and caspase-3 activity assayed by confocal microscopy (A) and FACS analysis (B). Scale bars: 20 μm

In keeping with these findings the percentage of TUNEL-positive apoptotic cells was increased by H+R and significantly decreased by $Mn^{II}(Me_2DO2A)$ (Figure 17)

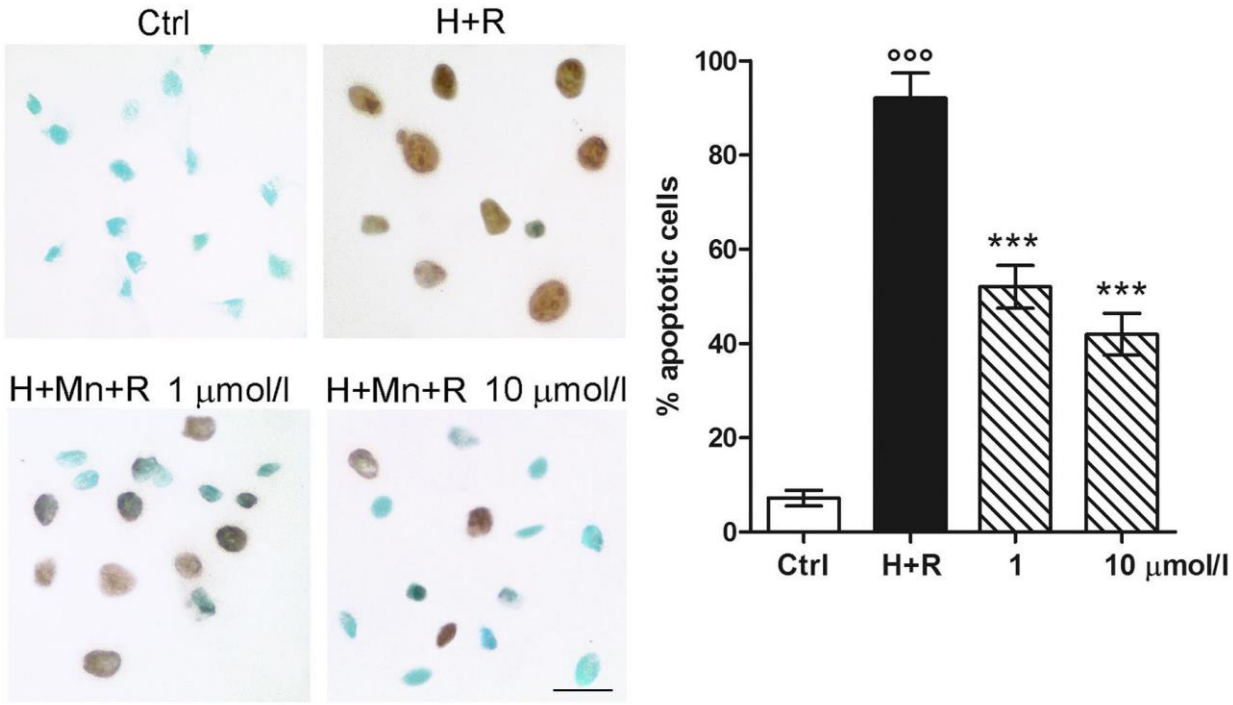

Fig.17 Evaluation of apoptosis in H9c2 cells by TUNEL assay. Scale bar: 10 μm.). Significance of differences: °°°p<0.001 vs. control; *p<0.05 and ***p<0.001 vs. H+R.

$Mn^{II}(Me_2DO2A)$ protects cardiac muscle cells from oxidative damage induced by H+R.

$Mn^{II}(Me_2DO2A)$ decreased H9c2 cell death by reducing the oxidative stress occurring at reoxygenation. In fact, determination of intracellular ROS and mitochondrial $O_2^{\bullet-}$ by loading the cells with the fluorescent probes H_2DCFDA and MitoSOX, respectively, showed that these oxidant species were markedly increased by H+R, whereas they were significantly reduced by 10 μmol/l and, at a lesser extent, 1 μmol/l $Mn^{II}(Me_2DO2A)$ (Figure 18 A,B).

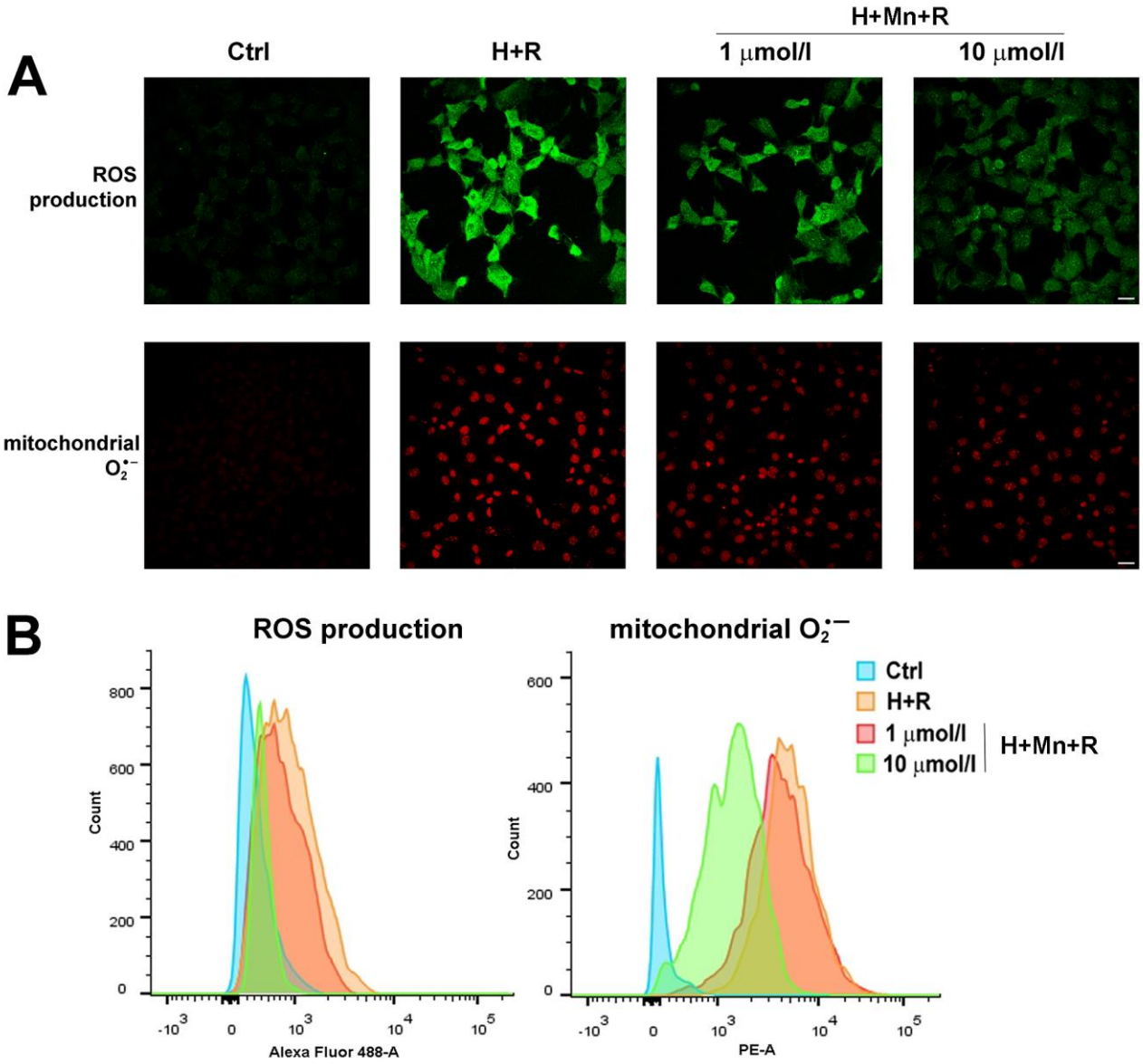

Fig.18 Evaluation of intracellular ROS and mitochondrial $O_2^{\bullet-}$ production by H_2DCFDA and MitoSOX assays by confocal microscopy (A) and and FACS analysis (B) Scale bar: 20 μm

In keeping with these findings, the levels of immunoreactive nitrotyrosine (NT), a marker of protein nitration which were enhanced upon H+R, were significantly reduced after the addition of 10 μmol/l $Mn^{II}(Me_2DO2A)$ at reoxygenation (Figure 19).

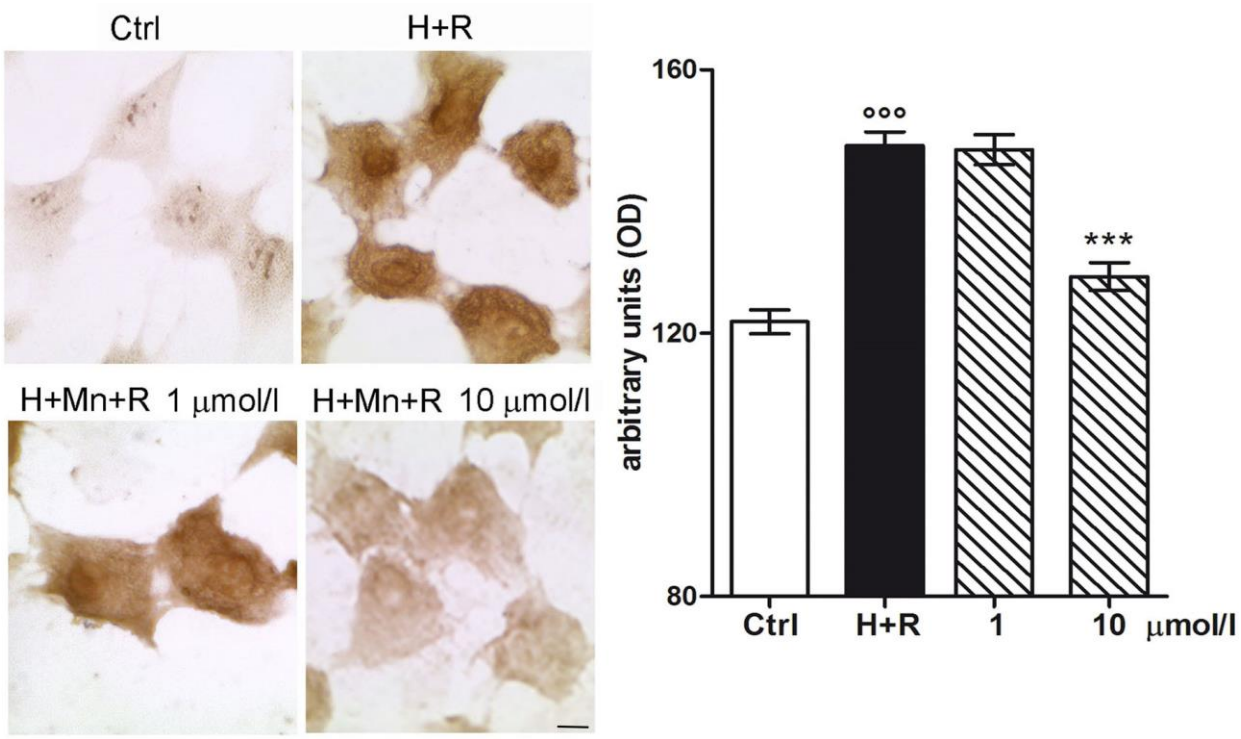

Fig. 19 Evaluation of H9c2 cell nitroxidative stress by immunoreactive nitrotyrosine. Scale bar: 10 μm. Significance of differences: °°°p<0.001 vs. control; *p<0.05 and ***p<0.001 vs. H+R.

Similar findings were observed in the experiments performed to evaluate the degree of DNA oxidation (Figure 20): in this instance, 10 μmol/l $Mn^{II}(Me_2DO2A)$ significantly reduced the levels of 8-OHdG in H9c2 cell lysates.

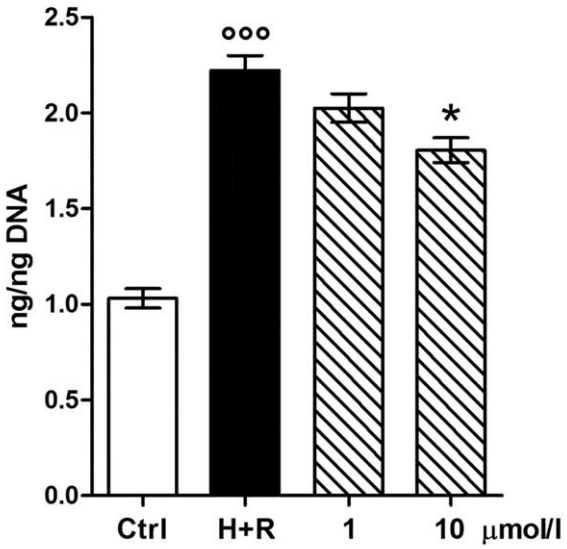

Fig.20 Evaluation of oxidative stress by 8-OHdG assay. Significance of differences: °°°p<0.001 vs. control; *p<0.05 and ***p<0.001 vs. H+R.

$Mn^{II}(Me_2DO2A)$ has no toxic effects on cardiac muscle cells

As shown by the MTT assay (Figure 21), $Mn^{II}(Me_2DO2A)$ had no toxic effect on H9c2 cells, even at 10-100-fold higher concentrations (100 μmol/l) and for longer exposure times (24 h) than those displaying significant biological effects. Similarly, $Zn^{II}(Me_2DO2A)$ (0.1-100 μmol/l) was also innocuous for the cells (Figure 21).

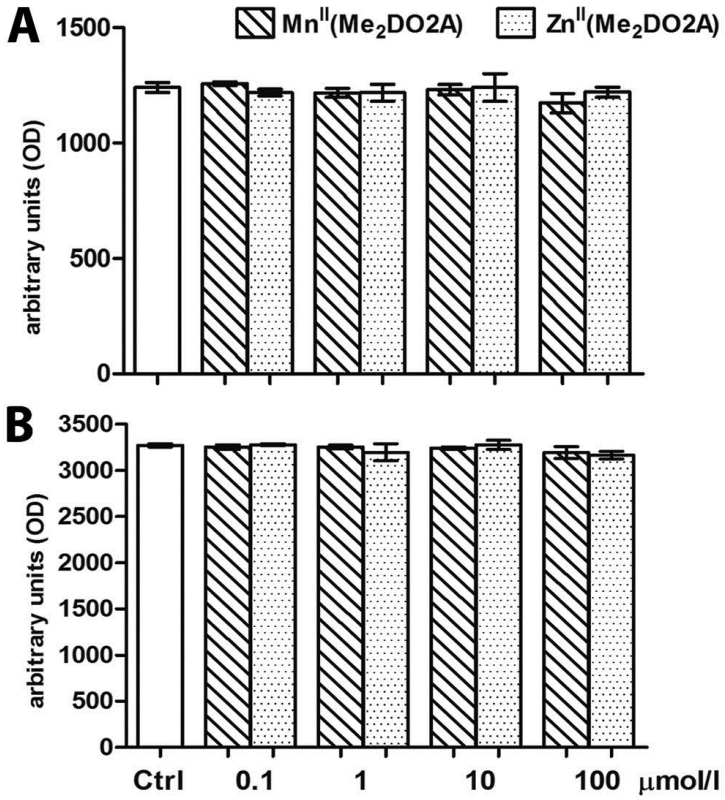

Fig.21 Evaluation of the toxicity of $Mn^{II}(Me_2DO2A)$ and $Zn^{II}(Me_2DO2A)$ on H9c2 cells by the MTT assay.

Discussion

Formation of reactive oxygen and nitrogen species and the resulting nitroxidative stress are the most invoked pathogenic mechanisms of ischemia–reperfusion-dependent diseases (Jaeschke and Woolbright, 2012; Murphy 2009; Poyton et al., 2009; Castello et al., 2008; Galkin et al., 2007) and justify the search for new drugs capable of limiting the over-production of $O_2^{\bullet-}$, $NO^{\bullet}$ and $ONOO^-$. In this context, previous studies have demonstrated that low molecular weight $NO^{\bullet}$ scavengers and SOD-mimetic Mn-containing porphyrins can preserve the function of isolated rat myocardial mitochondria subjected to hypoxia-reoxygenation and can protect the heart from ischemia-reperfusion injury in in vivo models (Robin et al., 2011; Masini et al., 2002). On a concurrent line of evidence, potentiation of $O_2^{\bullet-}$ decomposition capability of H9c2 rat cardiomyocytes by transfection with Cu/Zn SOD was found to increase their resistance to hypoxia-reoxygenation damage (Liu et al., 2013). The present cell culture model is intended to study the possible protection afforded by $O_2^{\bullet-}$ scavenging on cardiac muscle cells subjected to hypoxia-reoxygenation-induced nitroxidative stress. In the noted experimental conditions, the $O_2^{\bullet-}$ scavenger $Mn^{II}(Me_2DO2A)$, added at reoxygenation at low, micromolar concentrations, effectively prevented mitochondrial $O_2^{\bullet-}$generation, intracellular ROS generation, protein nitroxidation and oxidative DNA damage, thereby reducing apoptotic cell death, improving mitochondrial function and increasing cell viability. In a previous study, we have shown that $Mn^{II}(Me_2DO2A)$ has lipophilic properties which allow it to easily cross the cell membranes (Failli et al., 2009). This property suggests that this scavenger was able to attain $O_2^{\bullet-}$-neutralizing levels within the cardiac muscle cells and exert antioxidative effects close to the mitochondrial sited of $O_2^{\bullet-}$ generation, as suggested by the results of the MitoSOX and TMRM membrane potential assays. Moreover, Mn^{II} forms a highly stable complex at physiological pH with the polyamine-polycarboxylate scaffold. Other metal cations present in the cellular environment, including Ca^{II}, Mg^{II} and K^{I}, give remarkably less stable complexes than the functional Mn^{II} ion. The high stability of the $Mn^{II}(Me_2DO2A)$ prevents both de-metallation of the complex upon Mn^{II} complexation by cellular chelating agents and trans-metallation reactions, due to complexation of the ligand to other metals present in the cellular medium. As a matter of fact, $Mn^{II}(Me_2DO2A)$ does not release Mn^{II} even in the presence of large excess of Ca^{II} and other metal ions and of broad variations of pH (at pH 6, Mn^{II} release is less than 5%) (Failli et al., 2009). These properties are particularly important in view of a possible pharmacological extension of $Mn^{II}(Me_2DO2A)$ to the protection of ischemic-reperfused heart *in vivo*: in fact, the injured myocardium undergoes an overload of Ca^{II} (Bourdillon et al., 1981), which may potentially compete with the active Mn^{II} centre of the compound, and prominent acidosis due

to metabolic impairment. Moreover, $Mn^{II}(Me_2DO2A)$ is less susceptible to inactivation by oxidative stress conditions than endogenous or exogenously administered SOD (Finkel, 2005; Liu et al., 2013). The possible molecular mechanism of $O_2^{\bullet-}$ scavenging by $Mn^{II}(Me_2DO2A)$ may consist of a catalytic cycle involving first oxidation of Mn^{II} to Mn^{III} by $O_2^{\bullet-}$ and then reduction of the resulting Mn^{III} complex by another $O_2^{\bullet-}$ to form the initial Mn^{II} compound (Failli et al., 2009; Cinci et al., 2010). Interestingly, Mn^{II} in aqueous solution is oxidized *via* a single-electron process with redox potential higher than that of natural Mn-SODs. This suggests that, in the cellular environment, $Mn^{III}(Me_2DO2A)$ reduction may occur upon reaction with $O_2^{\bullet-}$ as well as other cytoplasmic reductans. To confirm these assumptions, the antioxidative effects of $Mn^{II}(Me_2DO2A)$ were completely lost when inactive Zn^{II} was substituted for Mn^{II}. Taken together, these features allow $Mn^{II}(Me_2DO2A)$ to behave as an efficient $O_2^{\bullet-}$ scavenger. Of note, Mn^{II} pentaazamacrocyclic complexes have been shown to react with $ONOO^-$ and dismute $NO^{\bullet}$, albeit at lower rates than with $O_2^{\bullet-}$ (Filipović et al., 2010). Whether $Mn^{II}(Me_2DO2A)$ may also be able to remove harmful $ONOO^-$ and excess $NO^{\bullet}$, thereby increasing its antioxidant properties, remains a tantalizing matter for further investigation.

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1. **Boccalini G.**, Sassoli C., Formigli L., Bani D., Nistri S**.** (2014) Relaxin protects cardiac muscle cells from hypoxia/reoxygenation injury. Involvement of Notch-1 pathway. *FASEB J* pii: fj.14-254854
2. Nistri S., **Boccalini G.**, Bencini A., Becatti M., Valtancoli B., Conti L., Lucarini L., Bani D. (2014) A new low molecular weight MnII-containing scavenger of superoxide anion protects cardiac muscle cells from hypoxia/reoxigenation injury. *Free Radical Research* 28:1-28