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Attenuation of oxidative stress in HL-1 cardiomyocytes improves mitochondrial function and stabilizes Hif-1α

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Abstract
HL-1 cardiomyocytes were subjected to simulated hypoxia, in the presence of cobalt chloride, which resulted in reduction of cell viability and induction of DNA laddering, indicating the activation of the apoptotic cascade. In the presence of trolox, ascorbic acid, melatonin and the hybrid compound of trolox and lipoic acid (LaT 3a), cell viability was increased, with LaT 3a exhibiting the best effect. Antioxidant treatment restored ATP levels, abolished laddering of DNA, abrogated MPTP opening, Bax translocation to the mitochondria and cytochrome c release to the cytoplasm. Moreover, severe hypoxia, was found to destabilize hypoxia inducible factor-1α (Hif-1α) mRNA. Reduction of oxidative stress attenuated this effect, implying a possible anti-apoptotic action of the master regulator of hypoxia response. Our data suggest that antioxidants can maintain cell function and survival by inhibiting the mitochondrial apoptotic pathway and stabilizing Hif-1α.

Keywords: *ROS, hypoxia, apoptosis, cardiomyocyte, Hif-1α*

Abbreviations: *ROS, reactive oxygen species; LaT, lipoic acid-trolox; MPTP, mitochondrial permeabilization transition pore; Hif-1α, hypoxia inducible factor-1α; ETC, electron transport chain; VDAC, voltage dependent anion channel; ANT, adenine nucleotide transporter; HRE, hypoxia response element; VHL, Von Hippel Lindau; VEGF, vascular endothelial growth factor; ANP, atrial natriuretic peptide; ANF, atrial natriuretic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; αHIF, antisense Hif-1α*

Introduction

Nonspecific damage to lipids, proteins and DNA caused by overproduction of reactive oxygen species (ROS) is implicated not only in degenerative conditions such as aging, Parkinson's disease, Alzheimer's disease and other injuries such as ischemia-reperfusion, but also, during hypoxia [1]. Either by limiting the availability of oxygen at the end of the electron transport chain (ETC) or by inhibiting the activity of one complex within the ETC, hypoxia accelerates the rate of electron leak in the ETC, leading to oxidative stress [2]. Because of the continuous exposure of mitochondria to ROS, these organelles accumulate oxidative damage more rapidly than the rest of the cell [3]. The central role played by mitochondria in molecular events leading to alteration or loss of cellular function has resulted in many studies implying mitochondrial dysfunction caused by ROS with both necrotic and apoptotic cell death [4,5].

Increasing evidence suggests that hypoxia-induced ROS play a key role in promoting cytochrome c release from the mitochondria and cytochrome c in the cytoplasm triggers apoptosome formation and subsequent activation of the caspase cascade that ultimately leads to apoptosis [6]. The outer mitochondrial membrane contains a voltage dependent anion channel

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(VDAC), which associates with the adenine nucleotide transporter (ANT) and cyclophilin D at contact sites between the inner and outer mitochondrial membranes to form a large nonspecific pore, the mitochondrial permeability transition pore (MPTP) [7]. Under hypoxic conditions, elevated Ca^{2+} and overproduction of ROS may cause both conformational change in the complex [7,8] and altered levels of Bcl-2 family proteins [9]. Either opening of the MPTP, or pore formation in the outer mitochondrial membrane provokes loss of the proton motive force required for energy production resulting in decreased ATP levels [10] and this in itself may be potentially disastrous for the heart, which requires a high degree of oxidative phosphorylation to provide the energy required for contraction. However, opening of the MPTP with subsequent increase in permeability of the inner mitochondrial membrane results in matrix swelling and collapse of the outer mitochondrial membrane which may be sufficient to influence cell death by affecting the release of cytochrome c and other apoptotic factors from the mitochondrial intermembrane space [11].

In order to overcome hypoxia induced damage, cells activate adaptive mechanisms in an effort to overcome the hypoxic conditions. In most cell types, activation of genes essential for cell survival is mediated by hypoxia inducible factor-1 (HIF-1), a transcriptional complex that binds to the specific hypoxia response element (HRE) of target genes [12]. HIF-1 is a heterodimer composed of the rate limiting factor Hif-1α and the constitutively expressed Hif-1β, also called the aryl hydrocarbon receptor nuclear translocator. During normoxia, Hif-1α protein is expressed but is unstable. Rapid degradation by the proteasome results from the ubiqitination of the hydroxylated oxygen dependent degradation domain of the α-subunit by the product of the Von Hippel Lindau (VHL) tumor suppressor gene, while under hypoxic conditions, the VHL protein fails to recognize Hif-1α, allowing HIF-1 to accumulate [13–15]. In the ischemic myocardium, this mediates the transcription of the vascular endothelial growth factor (VEGF), adrenomedullin, endothelin-1 and atrial natriuretic peptide (ANP) and other genes with HIF-1 binding HREs in their 5′-flanking regions. Besides its role in hypoxia response, recent data support that HIF-1 is implicated in the fine tuning of the regulation of apoptosis by hypoxia [16]. The role that the key regulator of hypoxia plays in this hypoxia mediated programmed cell death is not entirely clear yet and controversial published data suggest both pro-apoptotic and anti-apoptotic action [17,18].

Previous studies in our lab support a cardioprotective effect of antioxidant compounds on ischemic-reperfused hearts using the Krebs perfused Langendorff model [19–21]. In addition, recent evidence indicates that, in association with the production of ROS, apoptosis is a dominant form of cardiomyocyte death [22,23]. According to this, the aim of the present study was to investigate whether ROS mediate signaling events during hypoxia induced cell death and to define their possible link to the mitochondria dependent apoptotic pathway. HL-1 cardiomyocytes were used as a model system as these cells continuously divide, spontaneously contract and maintain a differentiated adult cardiac phenotype in culture [24]. In order to explore our hypothesis, mitochondrial homeostasis was tested by determining the ability of antioxidant compounds to prevent ATP depletion, disruption of the right balance between anti and pro-apoptotic proteins of the Bcl-2 family, MPTP opening and finally cytochrome c release. Finally, it was explored if Hif-1α transcripts are affected by the presence of antioxidant molecules under the same conditions. Our results suggest that scavenging of ROS can maintain cell function and survival by preserving the integrity of the mitochondria and stabilizing Hif-1α mRNA.

Materials and methods

Cell culture

HL-1 cells were grown in fibronectin-gelatin coated T75 flasks containing Claycomb medium (JRH Biosciences) supplemented with 10% fetal bovine serum (JRH Biosciences), 100 U/ml penicillin (PAA Laboratories), 100 μg/ml streptomycin (PAA Laboratories), 2 mM L-glutamine and 0.1 mM norepinephrine (Sigma). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 until 80–90% confluence. Hypoxia was stimulated by the presence of $CoCl_2$ (0–400 μM) for 24 h to the culture medium. The antioxidants used were trolox, ascorbic acid, melatonin and a hybrid compound of lipoic acid and trolox (LaT 3a) [21]. Cells were preincubated with the compounds for 24 h before the addition of $CoCl_2$ and were present during hypoxia at different concentrations.

Evaluation of cell survival

Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) incorporation and reduction by the succinic dehydrogenase of viable cells to an insoluble violet formazan product. HL-1 cells were seeded in 96-well culture plates at a density of 10^4 cells per well and when necessary, were pretreated with antioxidants for 24 h which were present throughout the hypoxic period. 20 μl MTT (stock solution 2.5 mg/ml in PBS) were added to each well for the last 4 h of hypoxic treatment. The medium was then aspirated and the cells lysed by addition of 100 μl 0.1 M HCl in isopropanol. The optical density of each sample was measured in an ELISA microplate reader (DENLEY) using a 540 nm filter.

Trypan blue exclusion

Following treatments, the medium in each plate was collected in a centrifuge tube. Cells attached to wells

were removed by trypsinization and pooled with the medium. Cells were centrifuged at 250 g for 5 min, and the pellet resuspended and incubated in PBS with 0.1% trypan blue for 5 min. Death rate was determined as the percent of dead cells over the total number of cells. A total number of 200–400 cells were counted under a microscope (Olympus CK2) by two investigators without knowledge of the respective treatments, and the mean was used for statistical analysis.

DNA ladder detection

Both attached and floating cells of HL-1 ($\sim 10^7$) were centrifuged (1200 rpm, 5 min) and washed with PBS. The pellet was resuspended in 600 μl buffer (100 mM Tris–HCl, pH 8, 200 mM NaCl, 10 mM EDTA) and after addition of equal volume of digestion buffer (100 mM Tris–HCl, pH 8, 200 mM NaCl, 10 mM EDTA, 0.4% SDS and 200 μg proteinase K) the cell lysate was vortexed and incubated at 55°C for 16 h. Subsequently, DNA was extracted with phenol–chloroform and RNase (20 μg/ml) was added to the aqueous phase. After incubation for 30 min at 37°C, DNA was precipitated with ice-cold ethanol and resolved in water. Yield and purity of DNA was determined by measuring the optical density at 260 and 280 nm. Electrophoresis was carried out on a 1.0% agarose gel and ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Determination of ATP

Cells ($2 \sim 4 \times 10^7$) were harvested, washed twice with PBS and maintained at liquid nitrogen for 30 min. Cells were lysed with 10% $HClO_4$ and centrifuged at 10,000 rpm for 10 min. Supernatants were removed carefully and were treated with 0.1 M Tris/0.1 M KOH until neutralization of the pH. ATP content was then determined during the transfer of phosphate group in glucose and subsequent reduction of NADP, in a reaction mixture containing 0.05 M phosphate buffer pH 7.5, 0.5 M glucose, 0.1 M $MgCl_2$, 1 mg/ml NADP, 10 mg/ml hexokinase and G-6PDH (700 units/ml).

Matrix swelling in isolated mitochondria

In a separate series of experiments, the potential effect of oxidative stress on mitochondrial matrix swelling was assessed spectrophotometrically in a suspension of isolated mitochondria from HL-1 cells subjected to hypoxia. Mitochondria were isolated by differential centrifugation. Briefly, $3 \sim 4 \times 10^7$ cells were harvested and washed twice with PBS. The pellet was suspended in 400 μl mitochondrial isolation buffer (MIB) (250 mM Sucrose, 20 mM K^+Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF and 1 mM DTT). After incubation at 4°C for 20–30 min cells were lysed using a Dounce homogenizer (40–50 strokes with pestle B). Homogenate was centrifuged for 5 min at 3500 *g* twice. The resulting supernatant was further centrifuged for 15 min at 10,000 *g* and the pellet (mitochondrial fraction) was resuspended in the isolation buffer. Protein concentration of both cytoplasmic and mitochondrial suspensions was determined using the BioRad Bradford assay.

Increases in mitochondrial matrix volume were determined as changes in the light scattering parameter of mitochondrial suspensions with the use of a spectrophotometer (Ultrospec 2000, Pharmacia Biotech). Light absorbance of the mitochondrial suspension (at 0.5 mg/ml) was measured at 540 nm for 20 min. The swelling effect was examined by measuring the drop of the absorbance. Passive swelling was induced with the addition of 0.25 mM $CaCl_2$ in the swelling buffer (10 mM Tris–MOPS, 0.05 mM EGTA, 5 mM Pyruvate, 5 mM Malate, 1 mM Pi–Tris, pH 7.4).

Immunoblot analysis

Proteins from both cytosolic and mitochondrial extracts (25 μg for cyt c and 100 μg for Bax and Bcl-xL) were separated by SDS-PAGE on 15% (w/v) acrylamide (for Bax and cyt c) or 10% (w/v) acrylamide (for Bcl-xL), 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were then incubated in TBS-T [20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween 20] containing 5% (w/v) nonfat dry milk for 1 h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies in TBS-T containing 5% BSA and then washed with TBS-T. Primary antibodies were anti-Bcl-xL (Cell Signaling, 1:1000), anti-Bax (Cell Signaling, 1:1000) and anti cyt c (Cell Signaling, 1:1000). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies [1:5000 dilution in TBS-T containing 1% (w/v) nonfat dry milk, 1 h, at room temperature]. The blots were washed again in TBS-T (4×5 min) and the bands were detected by using the enhanced chemiluminescence (ECL) reaction with exposure to X-OMAT AR film. The intensity of each immunoreactive band was estimated by densitometric quantification using Gel analyzer v1.0 software.

RNA isolation

Total RNA was isolated from HL-1 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNase-free water was used to dissolve RNA while yield and purity was determined by measuring the optical density at 260 and 280 nm. The integrity of the RNA was assessed by

formaldehyde–agarose gel electrophoresis followed by ethidium bromide staining.

RT-PCR

A total of 1.5 μg total RNA was reverse-transcribed by incubation with 20 μl reverse transcription mixture containing 5 pmole oligo $(dT)_{12\text{-}18}$ primer, 0.5 mM dNTPs, 1 × First-Strand Buffer, 10 mM DTT and 200 U Superscript II RNase H^- Reverse Transcriptase (Invitrogen) at 42°C for 50 min. The reverse transcriptase was inactivated by heating for 15 min at 70°C. PCR reaction was performed at a final concentration of 1 × PCR buffer, 0.2 mM dNTPs, 0.24 μM sense and antisense primers, 1.0 U Taq DNA polymerase (Finnzymes) using 2 μl cDNA to a total volume of 25 μl in a Tpersonal thermocycler (Biometra). Sense and antisense primers for Hif-1α and ANP are shown in Table I. The conditions of PCR amplification were 94°C 30 s, 60°C 30 s, 72°C 1 min (35 cycles) for Hif-1α, 94°C 20 s, 55°C 15 s, 72°C 30 s (27 cycles) for ANP and 94°C 30 s, 59°C 30 s, 72°C 30 s (25 cycles) for GAPDH. Amplified product was electrophoresed onto 2% agarose gels, stained with ethidium bromide, and the signal intensity was quantified using Gel analyzer.

Statistical evaluations

All data are presented as mean ± SD. Comparisons between control and treatments were performed using the Student's paired *t* test. A value of $P < 0.05$ was considered to be statistically significant.

Results

ROS mediate $CoCl_2$-induced cytotoxicity in HL-1 cells

HL-1 viability was reduced when exposed to $CoCl_2$ and the cytotoxicity of $CoCl_2$ was concentration dependent as shown by the MTT assay (Figure 1A). The survival rate of HL-1 was 70 ± 5% when the cells were treated with 200 μM $CoCl_2$ for 24 h and this concentration was selected for subsequent experiments. As shown in other studies too, $CoCl_2$ treatment along with the hypoxia response provokes an overproduction of ROS. To clarify whether ROS are a major cause of the observed cytotoxicity, HL-1 cells were treated with antioxidant compounds. As it is shown in Figure 2B, cell viability was increased when trolox, melatonin, or ascorbic acid were present in the medium at a concentration of 100 μM. However, the best cytoprotective action was exhibited by the hybrid compound of trolox and lipoic acid. LaT 3a at 10 μM increased cell viability in a statistically significant fashion to 88 ± 2%.

Same results were obtained when trypan blue exclusion assay was used for the determination of cell viability (Figure 1C). In this case, $CoCl_2$ induced cytotoxicity reached 25 ± 1% while antioxidant treatment reversed this effect, indicating the crucial role of oxidative stress in cardiomyocytes subjected to simulated hypoxia.

Effect of oxidative stress on intracellular ATP depletion under hypoxic conditions

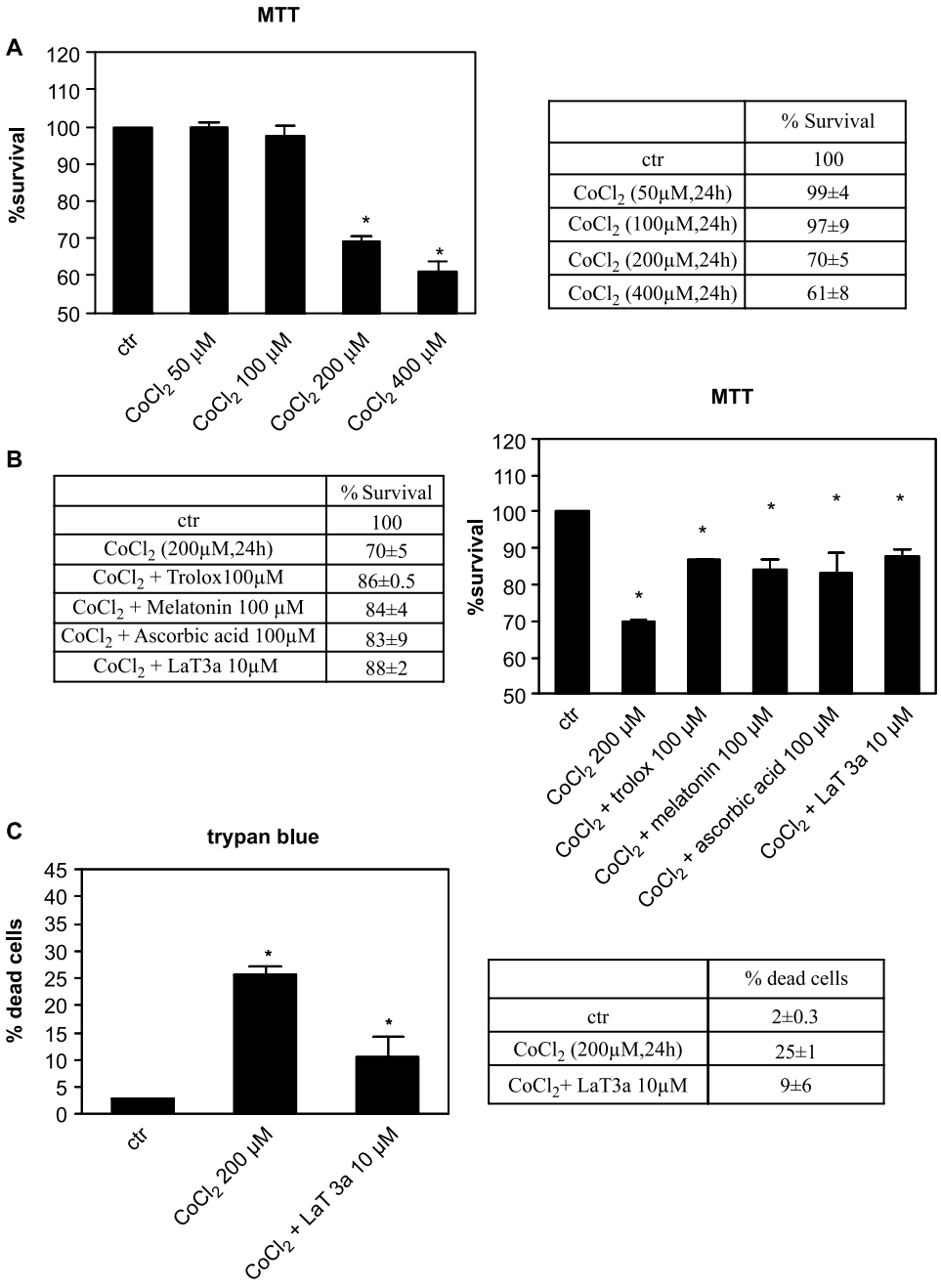
In order to characterize the early cellular events in hypoxic HL-1 cells and considering that capacity of MTT reduction is an index of mitochondrial function, energy status was tested. A significant decrease of the ATP values was noted after treatment with 200 μM $CoCl_2$ for 24 h as ATP content reached 70 ± 4% compared to cells grown in the physiological medium (Figure 2). Addition of the antioxidant LaT 3a at 10 μM, 24 h before and during the hypoxia period, partially restored ATP levels (84 ± 5%). Although both cell viability and ATP measurement indicate that HL-1 are tolerant under hypoxic conditions, data presented here indicate that cell death provoked by $CoCl_2$ treatment is in accordance with reduced ATP levels. Reverse in this effect by reducing oxidative stress supports the idea that ROS mediate $CoCl_2$ induced mitochondrial dysfunction.

Hypoxia induced apoptosis is partially attenuated after antioxidant treatment

Taking into consideration that pretreatment of HL-1 cells with the antioxidants increased cell viability and that ATP levels were not fully diminished during hypoxia, we next examined whether apoptosis is implicated in the $CoCl_2$ induced cell death. After exposure of HL-1 cells with 200 μM $CoCl_2$ for 24 h, typically pronounced DNA laddering was observed (Figure 3). However, treatment of cells with 100 μM

Table I. Sense and antisense oligonucleotides used for PCR amplification.

Primers	Sequence 5′-3′	Accession No.
Sense Hif-1α	CCTAACAAGCCGGGGGAGGAC	**Y09085**
Antisense Hif-1α	GATTCAACTGGTTTGAGGACA	**Y09085**
Sense ANF	GGGCTCCTTCTCCATCACC	**BC089615**
Antisense ANF	CTCCAATCCTGTCAATCCTACC	**BC089615**
Sense GAPDH	ACCACAGTCCATGCCATCAC	**BC083149**
Antisense GAPDH	TCCACCACCCTGTTGCTGTA	**BC083149**



	% Survival
ctr	100
$CoCl_2$ (50µM,24h)	99±4
$CoCl_2$ (100µM,24h)	97±9
$CoCl_2$ (200µM,24h)	70±5
$CoCl_2$ (400µM,24h)	61±8

	% Survival
ctr	100
$CoCl_2$ (200µM,24h)	70±5
$CoCl_2$ + Trolox100µM	86±0.5
$CoCl_2$ + Melatonin 100 µM	84±4
$CoCl_2$ + Ascorbic acid 100µM	83±9
$CoCl_2$ + LaT3a 10µM	88±2

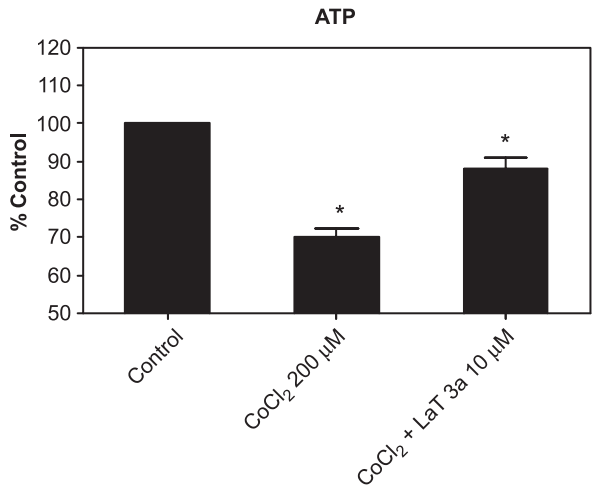
	% dead cells
ctr	2±0.3
$CoCl_2$ (200µM,24h)	25±1
$CoCl_2$+ LaT3a 10µM	9±6

Figure 1. Effect of $CoCl_2$ treatment on cell viability in the presence or not of compounds with well established antioxidant action. (A) HL-1 cells were subjected to hypoxia by adding into the medium increasing doses of $CoCl_2$ (0–400 µM) for 24 h. Cell viability was assessed by MTT assay. (B) Cells were preatreated with trolox (100 µM), melatonin (100 µM), ascorbic acid (100 µM) and LaT 3a (10 µM) for 24 h and then were made hypoxic (200 µM $CoCl_2$, 24 h) in the presence of the antioxidants. Cell viability was assessed by MTT assay, while in (C) cell cytotoxicity was measured by trypan blue exclusion assay. Results are expressed as the mean ± SD ($n = 3$) ($^*P < 0.05$).

trolox inhibited $CoCl_2$ mediated apoptosis (Figure 3A). The same effect was observed when LaT 3a was added in the medium at the concentration of 10 µM (Figure 3B), which fits with the powerful cytoprotective action exhibited by LaT in the survival tests. These findings indicate that oxidative stress during hypoxia can decrease cell viability by promoting apoptotic events rather than necrotic.

ROS mediate hypoxia induced MPTP opening and cyt c release

Cardiac myocytes have a very large energy requirement and mitochondria comprise a significant part of the intracellular volume within a cardiac myocyte. As both MTT assay and ATP levels indicate dysfunction of mitochondria, we next focused on the intrinsic mitochondrial apoptotic pathway. Regulation of the



	% ATP
ctr	100
$CoCl_2$ (200 μM,24h)	70±4
$CoCl_2$ + LaT3a 10 μM	84±5

Figure 2. Effect of hypoxia-induced ROS on cellular ATP levels. HL-1 were pretreated with LaT 3a (10 μM) for 24 h and then were made hypoxic in the presence of the antioxidant. Cells were collected for whole cell ATP measurement as described under "Materials and methods". ATP values are expressed as percent of control. All values are means ± SD ($n = 3$) ($^{\star}P < 0.05$).

mitochondrial permeabilization transition pore plays a key role in maintaining the impermeability of the organelle to all but a few selected metabolites, thus helping to maintain the membrane potential, which drives the ATP synthesis during oxidative phosphorylation. Therefore, our aim was to determine whether ROS were implicated in MPTP opening, which is considered as an early event in the apoptotic cascade. Since swelling of mitochondria is the main consequence of MPTP opening the swelling effect on intact mitochondria was examined by measuring the absorbance of the mitochondria for 20 min at 540 nm after addition of 0.25 mM Ca^{2+}. Figure 4A shows the drop in the absorbance due to increased mitochondrial swelling among the study group for the first 5 min. The drop was greater in mitochondria derived from cells treated with 200 μM $CoCl_2$ for 24 h compared to cells subjected to middle hypoxia (200 μM $CoCl_2$ for 6 h). In contrast, mitochondria from cells treated with the antioxidant LaT 3a seemed to be more resistant to the swelling phenomenon. These results are indicative that in prolonged hypoxia ROS play a crucial role in the opening of MPTP as LaT can retard this effect.

Cytochrome c release is a key event in the activation of the intrinsic apoptotic pathway. Once released to the cytosol, it interacts with Apaf-1 and pro-caspase-9 to form the apoptosome that ultimately activates caspase-9 and the subsequent caspase-3 activation leads to the final phase of apoptotic cell death. To determine if opening of the MPTP provokes cytochrome c release, its levels both into mitochondria and cytoplasm were determined. During normoxia the protein is detected mainly in the mitochondrium, while under hypoxic conditions (200 μM $CoCl_2$ for 24 h), is detected also in the cytoplasm. Scavenging hypoxia induced ROS by adding LaT 3a seems to provoke efficiently the release of cyt c to the cytoplasm (Figure 4B).

Antioxidant treatment impairs tranlslocation of Bax to the mitochondria in prolonged hypoxia while levels of Bcl-xL are unaffected

Apoptosis through the mitochondrial pathway is regulated by the right balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Beyond the action of the antioxidant compound in the cyt c levels, we wanted to test if an antiapoptotic Bcl-2 family protein, Bcl-xL, participates in the partial inhibition of MPTP opening during antioxidant treatment of the cells. Although bibliographic data suggest that Bcl-xL regulates MPTP opening, immunoblot analysis of

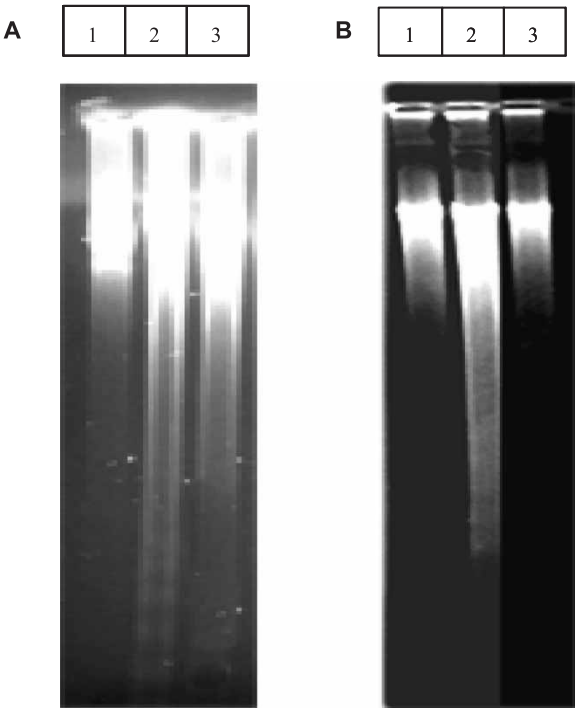


Figure 3. $CoCl_2$-induced DNA laddering and effect of antioxidants on hypoxia-induced apoptosis. HL-1 were grown for 24 h in medium with $CoCl_2$ (200 μM). In (A) 100 μM trolox was added 24 h before the simulated hypoxia and it was present during the hypoxic period. The number on the lanes represent: (1) control, non treated cells, (2) 200 μM $CoCl_2$, (3) 200 μM $CoCl_2$ in the presence of 100 μM trolox. In (B) 10 μM of the hybrid compound of trolox and lipoic acid was used as an antioxidant. The number on the lanes represent: (1) control, non treated cells, (2) 200 μM $CoCl_2$, (3) 200 μM $CoCl_2$ in the presence of 10 μM LaT 3a. Cells were harvested, genomic DNA was isolated and run on 1% agarose gel. Results are representative from three separate experiments.

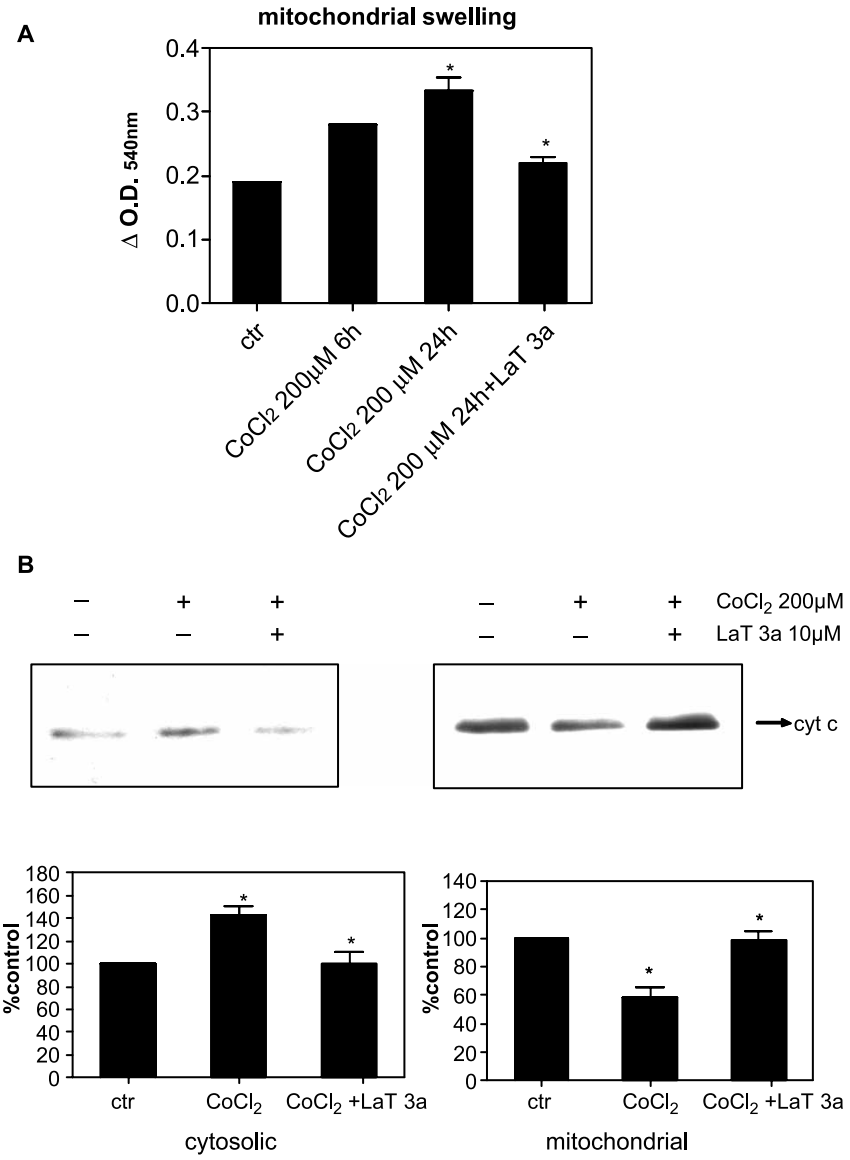


Figure 4. Effect of antioxidant treatment both on the prevention of mitochondrial matrix swelling in isolated mitochondria and on cyt c release to the cytoplasm. In (A) decrease in light absorbance reflects mitochondrial matrix swelling after addition of 0.25 mM Ca^{2+}. Mitochondria from cells subjected to severe hypoxia (200 μM $CoCl_2$, 24 h) are more sensitive to matrix swelling comparing to cells subjected to middle hypoxia (200 μM $CoCl_2$, 6 h). Scavenging of ROS inhibits mitochondrial swelling. In (B) cytoplasmic and mitochondrial cell extracts from hypoxic HL-1, in the presence or absence of the antioxidant compound LaT 3a, were immunoblotted for cyt c. Representative blot is shown in the upper panel, while densitometric analysis of the panel is provided in the lower panel expressed relative to controls. Data are presented as mean ± SD ($n = 3$) (*$P < 0.05$).

Bcl-xL revealed no participation of this protein under the examined conditions (Figure 5B). However, $CoCl_2$ treatment triggered Bax translocation to mitochondria. Diminishing the occurrence of oxidative stress by adding a hybrid compound of trolox and lipoic acid significantly blunted localization of Bax into mitochondrial fraction (Figure 5A). This result states that under hypoxic conditions ROS affect interaction between Bax and MPTP leading to cytochrome c release and apoptosis initiation.

Reduction of Hif-1α mRNA during prolonged hypoxia is reversed by decreasing oxidative stress

HIF-1 is an important transcription factor that is a crucial regulatory element in sensing hypoxic conditions and integrating an adapted response via gene expression of oxygen-sensitive enzymes and cofactors. Besides the role played by HIF-1 in the adaptation to hypoxia, recent data describe a possible role for HIF-1 in the modulation of apoptosis (for review 14). When HL-1 cells were subjected to prolonged hypoxia (200 μM $CoCl_2$) for

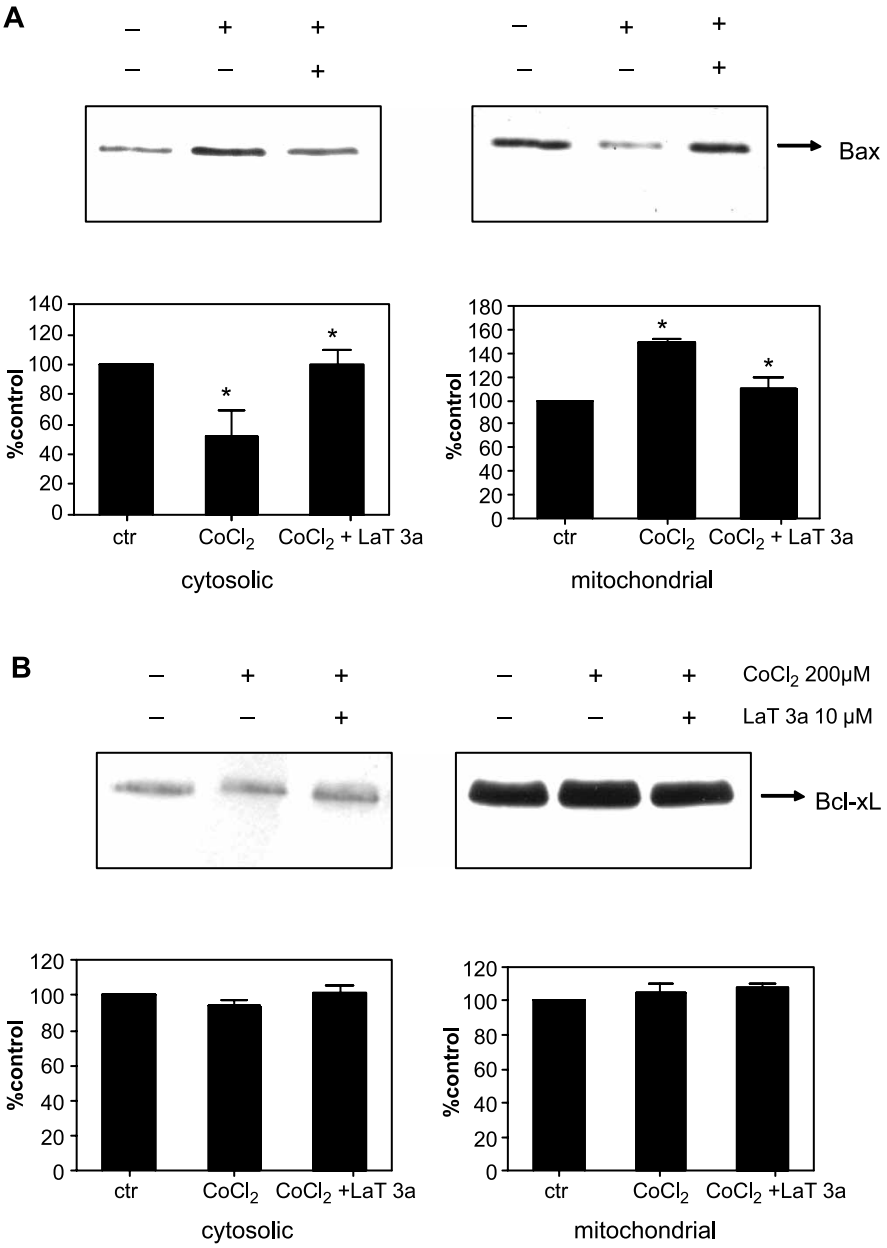


Figure 5. ROS mediate $CoCl_2$ induced Bax translocation to the mitochondria, without affecting Bcl-xL. Both cytoplasmic and mitochondrial cell extracts from hypoxic HL-1, in the presence or absence of the hybid antioxidant compound, were immunoblotted for Box (A) and Bcl-xL (B). Representatives blots are shown in the upper panels, while densitometric analysis of the panel is provided in the lower panels expressed relative to controls. Results are means ± SD for three independent experiments ($^{\star}P < 0.05$).

16 or 24 h, Hif-1α mRNA seems to be destabilized compared to control cells or cells subjected to middle hypoxia (Figure 6A). On the contrary, when cells were pretreated with the synthetic compound LaT 3a, Hif-1α transcripts were similar to control levels (Figure 6B). As recent studies suggest an implication of HIF-1 in apoptosis, we can propose that antioxidant treatment protects hypoxic HL-1 cells by stabilizing Hif-1α transcripts and probably enabling cells to activate genes which lead to a better adaptation under hypoxic conditions. Since it was found that ANP gene promoter is activated by hypoxia through direct action of HIF-1, our goal was to investigate if ANP gene is up-regulated under these experimental conditions and participates in the protection of HL-1 cells. However, RT-PCR analysis revealed that ANP transcripts remain stable (Figure 7A, B) implying that other target genes are probably affected by Hif-1α mRNA stabilization.

Discussion

Hypoxia is implicated in many common human diseases such as stroke, cardiac ischemia or cancer [25]. This explains the great interest of researchers in unraveling the molecular events during hypoxic conditions. Numerous studies have reported activation of the mitochondrial apoptotic pathway not only in different

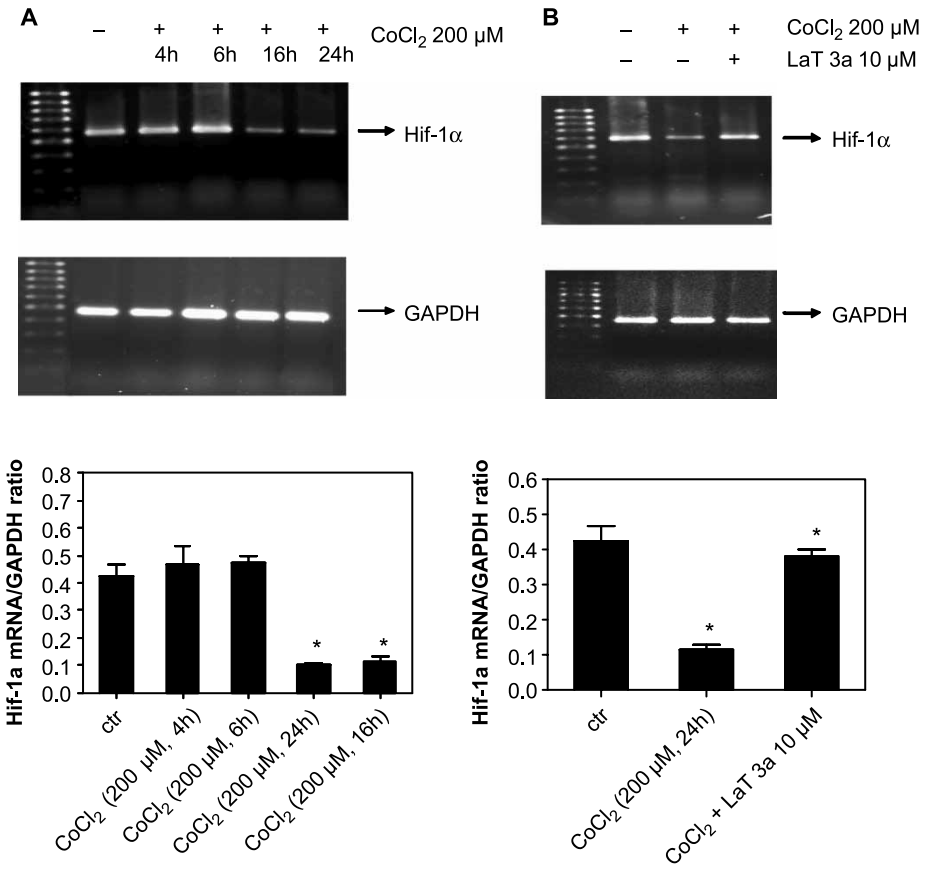


Figure 6. Prolonged hypoxia promotes destabilization of Hif-1α mRNA. (A) HL-1 were treated with $CoCl_2$ (200 μM) for different time periods (4, 6, 16 and 24 h). (B) The antioxidant compound LaT 3a, when used, was added 24 h before addition of $CoCl_2$ and was present through the hypoxic period. Semiquantitative RT-PCR was performed using specific primers for Hif-1α and GAPDH (see Table I for primer sequences). DNA products were visualized with ethidium bromide on 2% agarose gels. Representative images from a single experiment are shown in the upper panels. Hif-1α products were normalized to GAPDH as an internal control for densitometric analysis. Results are means ± SD for three independent experiments (*$P < 0.05$).

cardiac models [26], but also in cultured cardiac myocytes in models of hypoxia [27,28]. At the same time studies have reported that oxidative stress can induce apoptosis in cardiac myocytes and that overproduction of ROS can alter mitochondrial homeostasis [29]. Although antioxidant treatment exerts beneficial effects in a variety of experimental systems [30], the exact role played by ROS in hypoxia induced cell death has not yet been elucidated. Taking into consideration that cardiomyocytes are terminally differentiated and once destroyed they are not replaced, exploring cell death pathways is very important in understanding the development of heart diseases.

In the present study, we examined the hypothesis that oxidative stress during hypoxia plays a key role in cell death and we tried to explore the implication of the intrinsic mitochondrial pathway in this response. For this purpose HL-1 cells were used as a model cell culture system [24,31] and cobalt chloride was used as a hypoxia mimic. It is well established that $CoCl_2$ both regulates a similar group of genes comparing to hypoxia [32] and promotes over production of ROS [33,34]. Our results demonstrate that oxidative stress reduces cell viability and provokes ATP depletion (Figures 1 and 2). Between the antioxidant compounds tested, it seems that the hybrid compound of trolox and lipoic acid exhibits the best cytoprotective action (Figure 1B.) and this gives an explanation for its use in most experimental procedures. This is in accordance with data both from our group [21] and others [35] which support that a better antioxidant defence is established after the combination of these two antioxidants. We also showed that reduction of cell death is due to attenuation of apoptosis, as scavenging ROS, produced by $CoCl_2$ treatment, decreases DNA fragmentation (Figure 3). To further support the idea that oxidative stress in cardiomyocytes subjected to simulated hypoxia triggers apoptosis and activates especially the intrinsic mitochondrial pathway, it was found that antioxidant treatment blocks opening of the MPTP (Figure 4A) and the release of cytochrome c into the cytoplasm (Figure 4B). Moreover, ROS mediate Bax translocation to mitochondria (Figure 5B), without affecting levels of an antiapoptotic protein of the Bcl-2 family, Bcl-xL (Figure 5A). Previous results examining the relationship between the MPTP and Bax in apoptosis have raised a

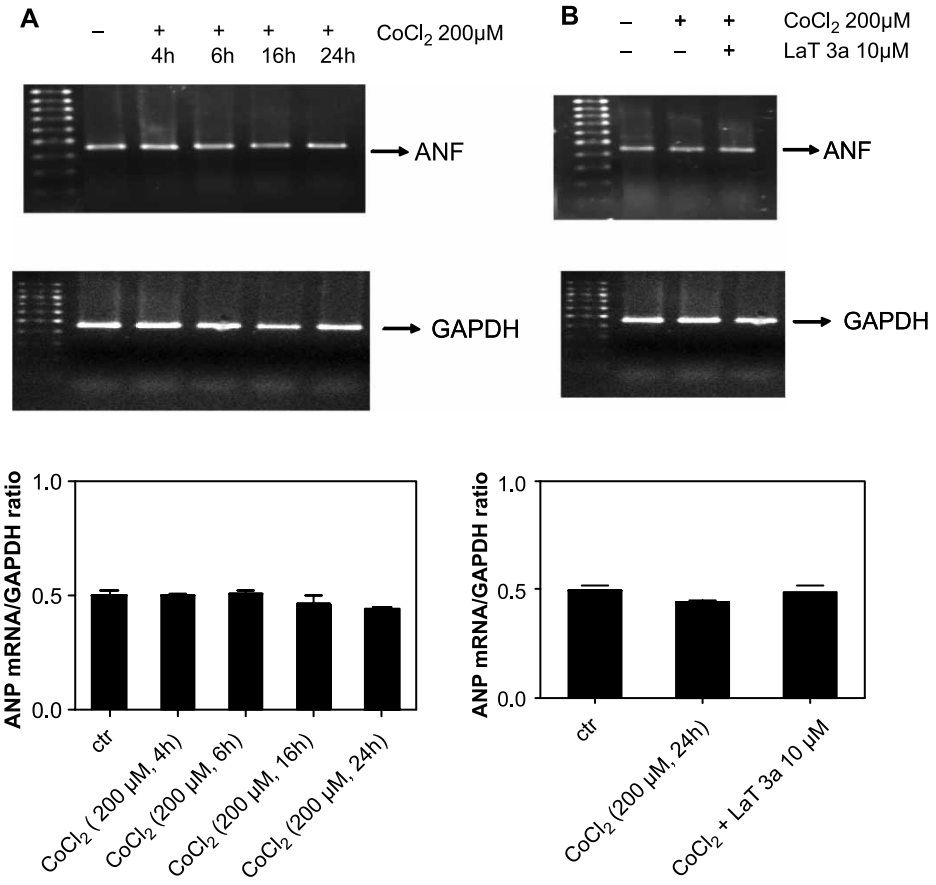


Figure 7. Destabilization of Hif-1α mRNA is not accompanied by change in ANF mRNA levels. (A) HL-1 were treated with $CoCl_2$ (200 μM) for different time periods (4, 6, 16 and 24 h). (B) The antioxidant compound LaT 3a, when used, was added 24 h before addition of $CoCl_2$ and was present through the hypoxic period. Semiquantitative RT-PCR was performed using specific primers for ANF and GAPDH (see Table I for primer sequences). DNA products were visualized with ethidium bromide on 2% agarose gels. Representative images from a single experiment are shown in the upper panels. ANF products were normalized to GAPDH as an internal control for densitometric analysis. Results are means ± SD for three independent experiments.

lot of controversy. There are published data providing evidence that either Bax or MPTP opening alone may be sufficient to induce cytochrome c release [36,37], while there is evidence for Bax interaction with VDAC, a component of the MPTP [38]. Recently, it was shown that MPTP triggers Bax translocation to mitochondria during neuronal apoptosis [39]. Our observation supports these later findings, indicating that in hypoxia-induced apoptosis both MPTP opening and Bax translocation happens. Furthermore, antioxidant treatment abrogates opening of the pore and decreases localization of Bax to the mitochondria, implying ROS as mediators of this response.

As a result of hypoxia, cells activate adaptive mechanisms which are in majority dependent on the transcription factor HIF-1 [12,40]. Recently, it was shown that hypoxic activation of atrial natriuretic factor (ANF) depends on HIF-1 and hypoxic induction of ANP is believed to contribute to increasing the oxygen tension in hypoxic tissues [41]. Nowadays, it is accepted that besides the role played by HIF-1 in hypoxia response, this transcription factor could modulate the apoptotic pathway, although contradictory results are emerging. Indeed, HIF-1 either by interacting with p53 [42] or by regulating the expression of Bcl-2 pro-apoptotic members such as Nip3 [43] and Noxa [44], renders cells sensitive to apoptosis. On the contrary, accumulating data suggest that HIF-1 can prevent apoptosis via expression of anti-apoptotic proteins. In fact, Piret et al. [45] demonstrated that HIF-1 dependent overexpression of Mcl-1 protects hypoxic cells against apoptosis, while Dong et al. described the overexpression of Bcl-xL in hypoxia selected cells resistant to cell death [46]. Therefore, it is of great interest the investigation of Hif-1α implication in the apoptotic pathway in $CoCl_2$ treated cardiomyocytes. Cardiac myocytes are not adapted to face long periods of hypoxia and thus, may have different adaptive mechanisms comparing to cancer cells, where hypoxia is a common phenomenon. Our results show no participation of ANF under the examined experimental conditions (Figure 7). On the contrary, RT-PCR revealed that in prolonged hypoxia (16 or 24 h) levels of Hif-1α mRNA are reduced, while in middle hypoxia, no change in Hif-1α mRNA is observed (Figure 6A). This finding is in

agreement with others which show that under severe hypoxia natural antisense of Hif-1α transcript (aHIF) can regulate Hif-1α mRNA by increasing Hif-1α mRNA instability via exposing AU-rich elements in the Hif-1α mRNA 3′-UTR [33,47]. In the presence of the antioxidant this effect is retarded implying that ROS may affect the mechanism of Hif-1α mRNA stabilization (Figure 6B). It is possible that in middle hypoxia Hif-1α, been regulated at the post transcriptional level, can mediate pro-survival responses, while in severe hypoxia, instability of the mRNA can lead to pro-death responses. This is consistent with the observation that lower levels of Hif-1α transcripts, after treatment with 200 μM $CoCl_2$ for 24 h, are associated with activation of the apoptotic pathway. Our data support an anti-apoptotic role for HIF-1, since ROS induce both apoptosis and decrease in Hif-1α mRNA. Although the exact role played by HIF-1 needs further elucidation, it is probable that the anti-apoptotic action is mediated by regulating the expression of Bcl-2 family proteins with pro-survival effects.

In summary, cobalt chloride, a hypoxic mimic agent, decreases HL-1 viability by activating the apoptotic cascade. ROS play a dominant role in this response as antioxidant treatment partially reverses this effect, possibly, through preservation of mitochondrial function. In the presence of antioxidants, both opening of the MPTP and translocation of Bax to mitochondria are blocked, with subsequent retention of cytochrome c into mitochondria, preventing activation of the apoptotic pathway in cardiac myocytes. In addition, stabilization of Hif-1α transcripts during antioxidant treatment associates with attenuation of apoptosis. These findings support the hypothesis that hypoxia via overproduction of ROS stimulates signaling pathways leading to cell death.

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