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$CoCl_2$-simulated hypoxia in skeletal muscle cell lines: Role of free radicals in gene up-regulation and induction of apoptosis

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Abstract

Since it was suggested that cobalt chloride ($CoCl_2$) could mimic the O_2 sensing role of mitochondria by increasing reactive oxygen species (ROS) generation during normoxia, we studied the correlation between $CoCl_2$-generation of free radicals and the induction of a hypoxic cellular response in myogenic cell lines. In both L6C5 and C2C12 cell lines, exposure to $CoCl_2$ induced an increase of intracellular oxidants, the accumulation of HIF-1α protein, and the expression of vascular endothelial growth factor (*VEGF*) and/or *iNOS* genes. On the other hand, only ascorbic acid, but not trolox, was effective in lowering the *$CoCl_2$* gene up-regulation. Neither the cytotoxicity nor the apoptosis induced by $CoCl_2$ in skeletal muscle cells were modified by culture supplementation with either ascorbic acid or trolox. Thus, $CoCl_2$ treatment of myogenic cell lines may represent a useful and convenient *in vitro* model to study gene modulation induced by hypoxia in skeletal muscle, although cellular loss induced by this metal may involve mechanisms other than HIF-1α stabilization. It is unlikely, however, that ROS would represent the main mediators of $CoCl_2$ effects on muscle cells.

Keywords: *$CoCl_2$, ROS, skeletal muscle cells, gene expression, apoptosis*

Introduction

In skeletal muscle cells, local tissue hypoxia is an important adaptive stress in exercise training; it determines, at the molecular level, the up-regulation of the regulatory subunit of hypoxia-inducible factor-1 (HIF-1) [1]. Possibly as a consequence of HIF-1 up-regulation, the mRNA levels of myoglobin, vascular endothelial growth factor (VEGF), and glycolytic enzymes, such as phosphofructokinase, increase in a hypoxia-dependent manner, paralleling increases in mitochondrial and capillary densities [2,3]. It has also been established that sustained exposure to severe hypoxia has detrimental effects on muscle structure, including a clear reduction in muscle fiber area [4]; furthermore, cellular signs of mitochondrial degradation products prevail under conditions of increased reactive oxygen species (ROS) formation [5]. Although some controversy still exists as to whether hypoxia results in an increase (or decrease) in ROS production [6], it appears that ROS could modulate the activity of HIF-1 and other redox-sensitive transcription factors [7,8], and that ROS production, through the modification of nucleic acids, proteins, or membrane phospholipids, may be responsible for hypoxia-induced cell death in various tissues [9–11]. In skeletal muscle, hypoxia-induced apoptosis could be responsible for cell loss under several physiological or pathological conditions [12], but the molecular

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mechanisms involved in this process, and the specific involvement of ROS has not been clearly shown.

Cobalt is one of the best-known chemical inducers of hypoxia-like responses [13,14]. Several reports have shown that, in cells from different origins, cobalt chloride ($CoCl_2$) can determine the stability of the regulatory subunit of HIF-1α, with consequent activation of erythropoietin, VEGF and other genes [15–17]. The mechanism involved in the metal's activation remains unclear. Cobalt may allow activation of the cellular oxygen sensor, as hypoxia does, by substituting for iron in the porphyrin ring resulting in very low to no affinity for oxygen [18], or through inhibition of HIF-1α degradation by proteasomes [19]. Alternatively, cobalt may directly enhance HIF-1α stabilization through ROS formation [20]. It is well known that metals can generate ROS through various mechanisms, including Fenton– and Haber–Weiss type reactions, which are the most common. Through ROS-mediated reactions, metals cause activation of nuclear transcription factors, as well as the induction of various signaling proteins, cell cycle arrest, and apoptosis (reviewed in [21]). Although it was demonstrated that Co^{2+} increases ROS generation and oxidative stress in cells [22,23], results conflict as to whether this would represent the stimulus for the hypoxic gene response. Chandel et al. [24] demonstrated in Hep3B cells that $CoCl_2$ activates transcription by stimulating ROS generation via a mitochondria-independent mechanism. Salnikow et al. [25] showed that the formation of ROS following exposure of human A549 lung cells to $CoCl_2$ is not involved in the activation of HIF 1-dependent genes. Moreover, while the activation of ROS-sensitive transcription factors has been implicated in the apoptosis induced by $CoCl_2$ in neuronal PC12 cells [26] and cardiomyocytes [27], the disturbance of the ubiquitin–proteasome pathway has been identified as a major explanation for $CoCl_2$-induced apoptosis in human alveolar macrophage cells [28].

To validate the $CoCl_2$-treatment of myogenic cultured cells as a useful and convenient *in vitro* model to study the specific responses of proliferating and post-mitotic skeletal muscle cells to hypoxia, and to verify the involvement of ROS generation in this process, we analyzed the cellular responses of cultured myoblasts or myotubes to $CoCl_2$ in terms of generation of oxidant species, up-regulation of hypoxia-related genes, and induction of cell death through apoptosis. In order to prove the contribution of the ROS scavenging systems (both endogenous or exogenous) in these processes, we performed our experiments in two cell lines; namely, the C2C12 (mouse) and L6C5 (rat) muscle cell lines, which greatly differ in terms of their redox homeostasis [29,30]. We also explored the ability of antioxidant compounds to prevent $CoCl_2$-induced modifications.

Materials and methods

Materials

Dulbecco's modified Eagle medium (DMEM) with high glucose concentration (4500 mg/l), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Cobalt chloride ($CoCl_2$), ascorbic acid 2-phosphate (AsAc), trolox (TRX) and colchicine were purchased from Sigma Chemical Co. (St Louis, MO, USA). $CoCl_2$, AsAc and TRX solutions were prepared just before addition to cultures.

Cell culture and treatments

Experiments were carried out in C2C12 (mouse, cat. CRL-1772) and L6C5 (rat, cat. AL00001) myoblast cell lines, which were purchased from ATCC (Manassas, VA, USA Manassas, VA, USA) and ICLC (Genoa, Italy), respectively. The L6C5 cell clone was derived from the L6 rat muscle cell line and is characterized by a significant differentiation ability [31]. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (GIBCO), and supplemented with antibiotics under a 5% CO_2–95% air atmosphere at 37°C. In the protocols concerning undifferentiated myoblasts, cells were treated and analyzed 48 h after seeding, within the subconfluent state; whereas, for differentiated myotubes, treatments were performed 4 days after reducing serum to 2%. At this time, differentiation of myoblasts into multinucleated myocytes, confirmed by light microscopy, correlates with the increased expression of myogenin, as previously described [29]. $CoCl_2$ (0–700 μM) was added to the cultures for the last 1–24 h before cell collection and analysis. When indicated, ascorbic acid 2-phosphate (AsAc), a stable compound that does not induce toxicity in cell culture media, and trolox were added at a 100 μM final concentration 24 h before the $CoCl_2$ treatment and maintained until the end of the culture period.

For each protocol, multiple experiments were performed independently and, unless otherwise specified, the mean values (± SD) among experiments are presented and discussed. Statistical comparison between means was performed through *t*-test and/or the Mann–Whitney rank sum *U*-test.

Intracellular ROS

Intracellular ROS generation was measured by using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes Inc., Eugene, OR, USA). After treatments, cells were washed twice with PBS, collected by trypsinization and incubated with 10 μM H_2DCF-DA for 20 min at

37°C in the dark. Radical formation was assessed by flow cytometry in a FACS Calibur Flow Cytometer (Becton Dickinson, CA, USA). H_2DCF-DA mean fluorescence was registered at 530 nm (bandwidth 30 nm) and excited at 488 nm using a 15 mW argon laser. One hundred thousand events were evaluated for each analysis.

Cell viability and cytogenetic analysis

The number of viable cells in culture was evaluated by the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) as previously described [29,30]. To obtain cytogenetic samples, colchicine (0.04 μg/ml) was added to myoblasts for the last 1.5 h of culture. Cells were then collected by trypsinization and submitted to hypotonic (KCl, 0.075 M) and fixative (methanol/acetic acid 3:1) treatments to obtain metaphase spreads. Slides were stained with Giemsa (5%), coded, and blindly analysed. The frequency of chromosomal aberrations (CA) was calculated as the number of CA on 100 metaphases observed in each experimental point.

Protein extraction and Western blot analysis

Cellular extracts were obtained from mechanically removed L6C5 and C2C12 myotubes that were washed twice in PBS and resuspended in lysis buffer (0.05 M Tris–HCl pH 7.6 containing 1% NP40, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM NaPPi, 2 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml each of leupeptin, pepstatin and aprotinin). After 20 min on ice, lysates were centrifuged at 14,000*g* for 15 min at 4°C and protein concentrations were determined by Bradford method. Protein extracts (30 μg/lane) were resolved by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Amersham). Membranes were probed with specific antibodies against HIF-1α (Novus-Biologicals) or actin (Sigma), followed by horseradish peroxidase-coupled secondary antibodies, and developed by a chemiluminescence-based detection system (ECL, Amersham). Representative blots from three independent experiments are shown.

VEGF ELISA assay

Since in our cellular model the FBS concentration in the medium could interfere with both skeletal muscle cell differentiation and assessment of secreted VEGF, we decided to evaluate the cytoplasmic amount of VEGF protein. Thirty microgram of total protein was used for each assay. VEGF concentration was determined using an ELISA kit (R&D Systems), following the manufacturer's instructions. Samples from two independently performed experiments were analyzed in duplicate.

RNA extraction and RT-PCR analysis

Cultures were harvested for RNA, as described by Chomczynski and Sacchi [32]. Total RNA (6 μg) was reverse-transcribed in a 40 μl final volume, in the presence of 100 pmol of random hexamer primers (pdN6) for 1 h at 42°C, using 200 U M-MuLV Reverse Transcriptase (M-Medical Genenco-Life Science, Italy), and following the manufacturer's recommendations. A PCR reaction was then carried out on 7 μl of the RT reaction using 1.25 U Taq polymerase (M-Medical Genenco-Life Science) in the presence of 15 pmol of each specific primer in a total volume of 35 μl. Amplification was performed for 34 cycles (1 min denaturing at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C) in a thermal cycler. The miNOS-specific primers were: miNOS forward 5′CTTGCCCCTGGAAGTTTCTCTT3′, miNOS reverse 5′GCTGGTAGGTTCCTGTTGTTTC3′; primers for GAPDH, used as a semiquantitative control, were: forward 5′ACCACAGTCCATGCCATCAC3′, reverse 5′TCCACCACCCTGTTGCTGTA3′. The expected amplified products were 518 bp long for miNOS. GAPDH mRNA gave rise to an amplified product of 452 bp, obtained by using the same amplification conditions, except that 5 pmol of each primer were used and the amplification was performed for 28 cycles.

When VEGF expression was analyzed, PCR conditions were the same as above, except for the annealing step, which was performed at 47°C. The VEGF primers were: VEGFfw 5′TCGGGCCTCCGAAACCATGA3′, and VEGFrev 5′CCTGGTGAGAGATCTGGTTC3′. The expected amplified products were 513 bp for $VEGF_{120}$, 585 bp for $VEGF_{144}$, and 645 bp for $VEGF_{164}$. Amplification products were analyzed by 1.2% agarose gel electrophoresis and visualized under UV illumination after staining with ethidium bromide.

Apoptosis

The induction of apoptosis by $CoCl_2$ was evaluated by changes in nuclear morphology, DNA fragmentation and caspase 3-activation. To determine the induction of apoptosis at the single cell level, cells were grown on coverslips and, after treatments, were fixed with paraformaldehyde (4% in PBS) and stained with Hoechst 33258 (Sigma Chemical Co.). Apoptotic cell frequency was evaluated as the percentage of cells/1000 cells scored that showed condensed/fragmented nuclei. For the DNA fragmentation analysis, cells were collected, washed with PBS, and incubated for 1 h at 56°C in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1% SDS, 10 mM NaCl) containing proteinase K (200 μg/ml). DNA was extracted with two rounds of phenol/chloroform (1:1) and chloroform extractions, then precipitated in

ethanol. Purified DNA was quantitated by spectrophotometric absorption at 260 nm. Total DNA (10 μg) was electrophoresed on a 1.5% agarose gel and visualized with an ultraviolet transilluminator. Caspase 3-like activity was measured using the CaspACE Assay Kit, Colorimetric (Promega), following the manufacturer's instructions. Briefly, floating and attached cells were collected by scraping into cold PBS; cells were then pelleted, rinsed in PBS, and lysed in 30–50 μl of lysis buffer. After two rounds of freezing and thawing, the cell lysates were incubated on ice for 15 min, and the supernatant fraction was collected after centrifugation at 15,000*g* for 20 min. Protein concentrations were measured by the Bradford method, and 50 μg of total protein was used for each reaction. Following 4 h of incubation at 37°C, caspase-3 protease activity was measured in a spectrophotometer at 405 nm. For the higher dose, the assay was performed either in the presence or absence of a pancaspase inhibitor (Z-VAD-FMK). Values were reported as protein concentration (pmol/total proteins) based on a standard curve constructed for each independent measurement.

Results

The dose-dependent increase of intracellular ROS induced by $CoCl_2$ in L6C5 and C2C12 myoblasts correlates with the different sensitivity of the two cell lines towards $CoCl_2$ cytotoxicity, which is not attenuated by exogenous antioxidants. To demonstrate that $CoCl_2$ treatment of skeletal muscle cells could result in the generation of ROS, we evaluated ROS production in L6C5 and C2C12 myoblasts exposed to different concentrations (50–500 μM) of $CoCl_2$ for 1, 8 or 20 h. In accordance with their lower redox state, L6C5 myoblasts showed higher endogenous ROS levels than C2C12 cells, as previously described [29], and higher $CoCl_2$-induced ROS (Figure 1A,B). In L6C5 myoblasts, the values of H_2DCF-DA fluorescence after treatment with $CoCl_2$ at >50 μM for 1 h were statistically different ($p < 0.01$) from those of untreated cultures. In C2C12 myoblasts, in contrast, a statistical enhancement of H_2DCF-DA fluorescence over the basal endogenous level could be detected only after a treatment with 200 μM $CoCl_2$. Treatments with $CoCl_2$ at 300 μM or higher yielded a similar pattern of ROS production in the two cell lines (Figure 1A), although a specific decrease of cell number was observed at these $CoCl_2$ concentrations in L6C5, but not C2C12 cells. In fact, the higher susceptibility of L6C5 myoblasts to the cytotoxic effect of $CoCl_2$ was evident when skeletal myoblasts were exposed to $CoCl_2$ for 8 or 20 h. In the prolonged treatments, the levels of $CoCl_2$-induced ROS, as evaluated by the enhancement of H_2DCF-DA fluorescence, remained almost unchanged over time and required higher $CoCl_2$ concentrations to be detected in C2C12 myoblasts (Figure 1B). Moreover, in L6C5 myoblasts,

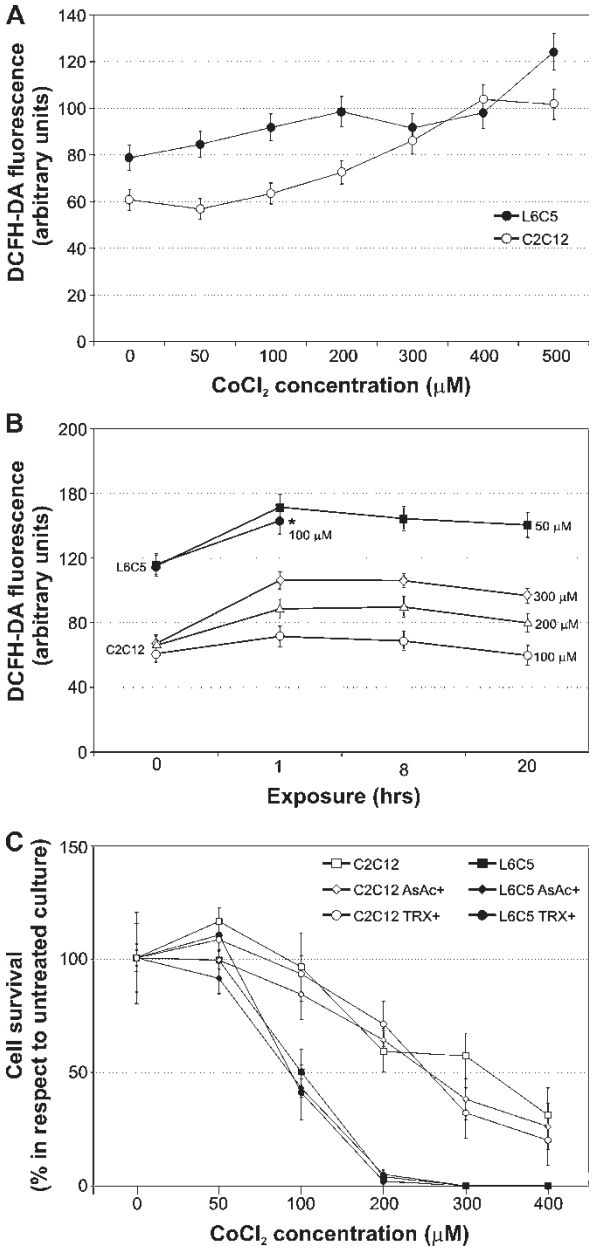

Figure 1. $CoCl_2$-induction of intracellular ROS and cytotoxicity in L6C5 and C2C12 myoblasts. Generation of intracellular oxidants in skeletal myoblasts treated with different concentrations of $CoCl_2$ for 1 h (panel A) or for 1, 8 and 20 h (panel B). *Results of H_2DCF-DA analysis after exposure for 8 or 24 h in L6C5 with $CoCl_2$ concentrations of 100 μM are not shown, since these treatments severely affected the cell number. (C) Effects of different treatments with $CoCl_2$, with or without antioxidants, on cell viability of L6C5 and C2C12 skeletal muscle cells. Cell viability is expressed as percentages compared to untreated culture.

all treatments for 8 or 20 h with $CoCl_2$ concentrations ≥ 100 μM affected the cell number to an extent that made H_2DCF-DA determination of ROS production unreliable. We thus analyzed the viability of L6C5 and C2C12 myoblasts after a prolonged exposure (24 h) to various $CoCl_2$ concentrations and, as shown in Figure 1C, we observed a cell line specific response. The statistical reduction to 50% viability ($p < 0.01$)

determined in L6C5 myoblasts at the concentration of 100 μM $CoCl_2$, was detected in C2C12 myoblasts only after a 24 h treatment with 300 μM $CoCl_2$, a drug concentration that resulted in 100% cell death in L6C5. To test if the enhancement of ROS induced by $CoCl_2$ could be specifically related to cell loss, exogenous antioxidants were added 24 h before the addition of $CoCl_2$ and maintained in the culture until cell collection. Neither ascorbic acid 2-phosphate (100 μM) nor trolox (100 μM) significantly attenuated $CoCl_2$-induced cytotoxicity in either of the skeletal muscle cell lines ($p > 0.05$), indicating that oxidative stress "per se" is not directly involved in this process.

In cultured myotubes, $CoCl_2$ induces the accumulation of HIF-1α and the expression of HIF-1α related genes, which are specifically inhibited by ascorbic acid 2-phosphate, but not by trolox. To test whether $CoCl_2$ can stimulate the activation of hypoxia-related genes in skeletal muscle cell lines, we decided to test the expression of HIF-1α and VEGF in differentiated myotubes after a 24 h treatment. Moreover, to assess the role of a ROS-scavenger activity in the $CoCl_2$-induced cellular response in skeletal muscle cells, we analyzed the effect of culture supplementation with AsAc and trolox reagents that are both able to modulate $CoCl_2$ up-regulation of HIF1-dependent genes [33].

We first tested if the differences in cell viability of L6C5 and C2C12 myotubes exposed to $CoCl_2$ for 24 h could interfere with gene up-regulation. The analysis showed that, as with H_2O_2 susceptibility [29], after differentiation, the sensitivity of L6C5 to the cytotoxic effect of $CoCl_2$ was analogous to that of C2C12 cells, and strongly reduced relative to proliferating myoblasts. Indeed, no effect on the viability of L6C5 and C2C12 myotubes was detected at concentrations lower than 500 μM. A combined treatment with AsAc 100 μM and $CoCl_2$ 500 μM was performed to verify the AsAc modulation of $CoCl_2$ cytotoxicity in myotubes, and, as with myoblasts, supplementation with AsAc did not decrease the cytotoxicity induced by $CoCl_2$ (Figure 2A).

As expected [34], Western blot analysis revealed an increase in HIF-1α protein in both L6C5 and C2C12 myotubes stressed for 24 h with $CoCl_2$ at 100 and 300 μM (Figure 2B), confirming that this metal can also induce a hypoxic response in skeletal muscle cells. In L6C5 extracts, the mAb utilized recognized multiple

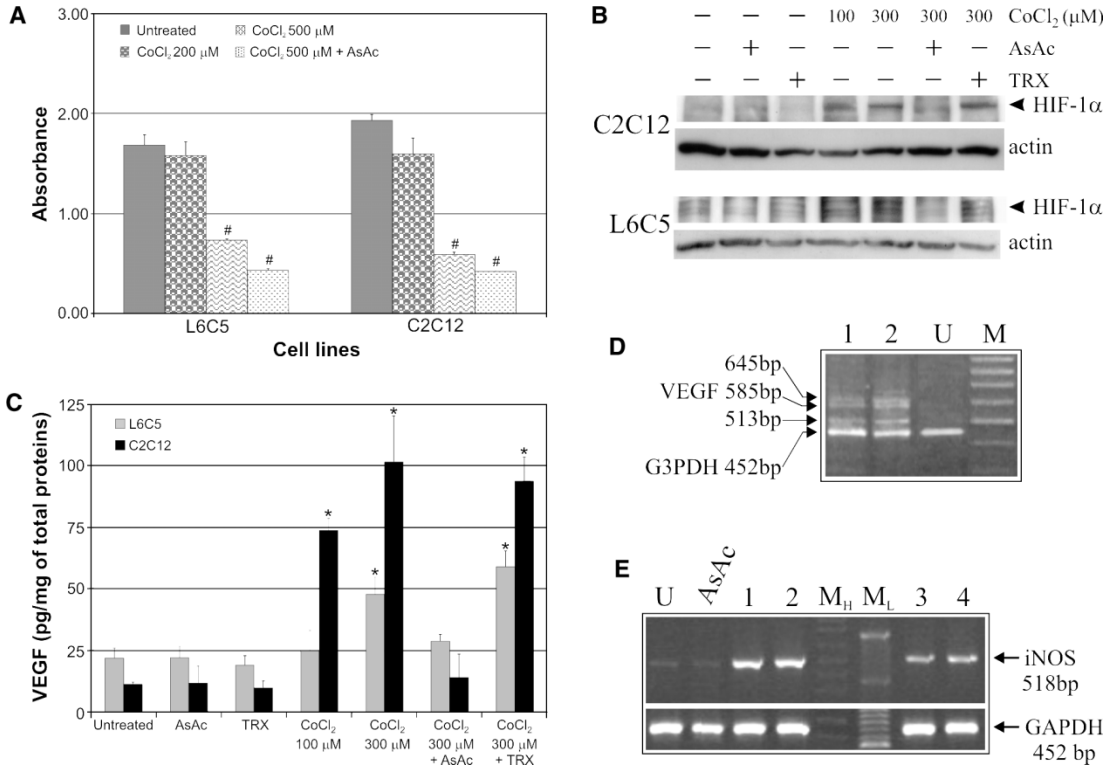

Figure 2. Effect of exposure of L6C5 and C2C12 myotubes to different concentrations of $CoCl_2$ on cell viability and up-regulation of hypoxia related genes. (A) Effects of different treatments with $CoCl_2$ on cell viability of L6C5 and C2C12 after cell growth in differentiation medium for four days. Cell viability is expressed as optical absorbance (OD). # = ($p < 0.01$). (B) HIF-1α expression in L6C5 and C2C12 myotubes treated with $CoCl_2$, with or without antioxidants. Actin was evaluated as a control for equal loading. (C) Intracellular accumulation of VEGF protein induced by $CoCl_2$ in L6C5 and C2C12 differentiated myotubes, with or without antioxidants. * = ($p < 0.05$). (D) RT-PCR analysis of VEGF expression induced by $CoCl_2$ in L6C5 differentiated myotubes. U, untreated cells; lane 1, $CoCl_2$ 100 μM; lane 2, $CoCl_2$ 300 μM. (E) RT-PCR analysis of expression of *iNOS* gene induced by $CoCl_2$ in C2C12 differentiated myotubes. U, untreated cells; lane 1, $CoCl_2$ 100 μM; lane 2, $CoCl_2$ 300 μM; lane 3, $CoCl_2$ 100 μM + AsAc; lane 4, $CoCl_2$ 300 μM + AsAc.

bands at 120 kDa representing post-translational modification of HIF-1α (Nova-Biologicals). Regarding VEGF accumulation, it showed a similar statistical increase at both of the $CoCl_2$ concentrations utilized in C2C12 myotubes (6.6- and 5.7-fold increase compared to untreated culture), whereas in L6C5 cells, the amount of VEGF was statistically increased only after treatment with the higher concentration of $CoCl_2$ (2.2-fold increase with respect to untreated culture) (Figure 2C). In order to further corroborate the data from our VEGF ELISA assay on L6C5 myotubes (where the goat anti-mouse VEGF antibody had only a 14% cross-reactivity towards rat VEGF isoforms), we also performed RT-PCR analysis to detect mRNA from the three major VEGF isoforms, 165, 144 and 120. The RT-PCR analysis (Figure 2D) demonstrated that $CoCl_2$ up-regulated *VEGF* gene transcription in rat myotubes at the same concentration range required in C2C12 myotubes.

After supplementation with exogenous antioxidants, we obtained a similar response in both cell lines. While the single treatment with AsAc (100 μM) or trolox (100 μM) did not modify the basal protein amounts, the presence of AsAc, but not trolox, inhibited the $CoCl_2$-induced enhancement of HIF-1α and VEGF in both L6C5 and C2C12 myotubes (Figure 2B,C). To further verify the effect of AsAc in the $CoCl_2$-induction of hypoxic response in skeletal muscle cells, we analyzed the expression of the *iNOS* gene in C2C12 myocytes, which is correlated to HIF-1α activity [35,36]. Although RT-PCR analysis did not allow an accurate quantitative evaluation, our results clearly showed that $CoCl_2$ specifically activated the transcription of the *iNOS* gene in C2C12 myotubes, and that this effect was reversed by ascorbic acid (Figure 2E).

$CoCl_2$ induces cell death through apoptosis in myogenic cells independently from AsAc supplementation with a high

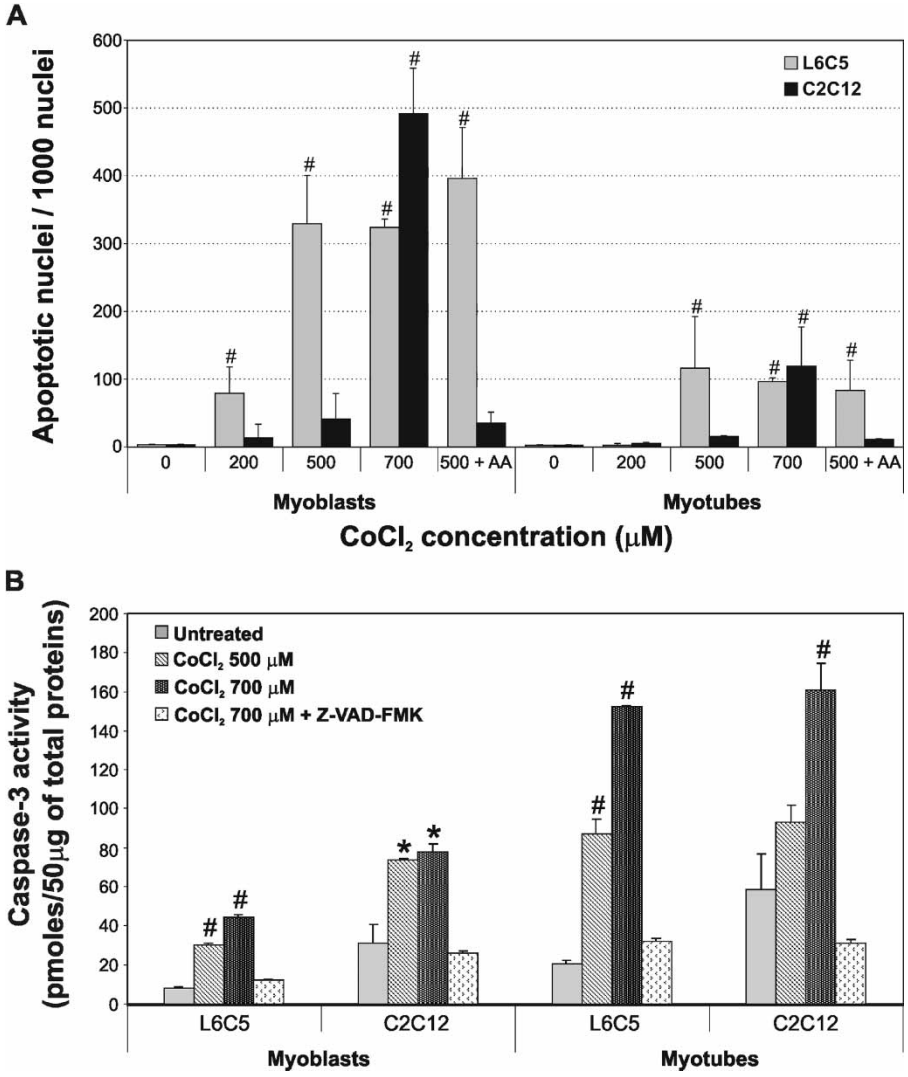

Figure 3. $CoCl_2$ induced nuclear fragmentation and caspase 3 activity in L6C5 and C2C12 skeletal muscle cells. (A) Nuclear fragmentation induced by $CoCl_2$ in L6C5 and C2C12 myoblasts and myotubes, alone or in combination with 100 μM AsAc. # = ($p < 0.01$). (B) Caspase 3-like protease activity in L6C5 and C2C12 proliferating and differentiated muscle cells. ($\star p < 0.05$; $\#p < 0.01$).

susceptibility of proliferating or resting mononucleated cells. The analysis of fragmented and/or condensed nuclei demonstrated that, in L6C5 myoblasts, the frequency of apoptosis was appreciable starting from the $CoCl_2$ concentration of 100 μM (3.2 vs. 0.2% in untreated culture), and showed a dose-dependent increase (7.9% at 200 μM; 32.9% at 500 μM; 32.4% at 700 μM). In C2C12 myoblasts, the induction of apoptosis was statistically significant only after a prolonged treatment with the highest $CoCl_2$ concentration of 700 μM (apoptotic nuclei, 49.2%) (Figure 3A). In differentiated cultures, the analysis of $CoCl_2$-induction of the apoptotic phenotype was evaluated as the net increase of apoptotic nuclei over the frequencies scored in untreated cultures, since high spontaneous apoptosis is normally correlated to the *in vitro* differentiation of these cell lines. The results showed a lower sensitivity of myocytes with respect to proliferating myoblasts (highest values, 11.6% for L6C5 and 11.9% for C2C12). Again, a specific sensitivity was observed in L6C5 cells at 500 μM $CoCl_2$, a concentration that was ineffective in C2C12 cells. As shown in Figure 4A,B, the apoptotic phenotype induced by $CoCl_2$ in L6C5 and C2C12 cells after differentiation involved almost exclusively mononucleated cells. Since these cells may contribute less to metabolic activity than multinucleated myotubes, their specific loss by apoptosis in L6C5 cultures could be underestimated by the MTS assay. This would explain the similarity between L6C5 and C2C12 detected through the cell survival assay in differentiated cultures. Since AsAc is known to counteract ROS-induced apoptosis in these cells [27,30], we analyzed the effect of AsAc supplementation on $CoCl_2$-induced apoptosis. As for cell survival, and in contrast to the results about HIF-1α modulation, ascorbic acid 2-phosphate was not effective in lowering the $CoCl_2$-induced nuclear fragmentation in either L6C5 and C2C12 myoblasts or myotubes (Figure 3A).

The evaluation of caspase 3 activity confirmed that the cell loss in skeletal muscle cells resulting from prolonged exposure to $CoCl_2$ was correlated to apoptosis. In both L6C5 and C2C12 myoblasts and myocytes, treatment with $CoCl_2$ 700 μM for 24 h resulted in a statistical increase of caspase 3 activity ($p < 0.01$) relative to untreated cultures (Figure 3B). After treatment with 500 μM $CoCl_2$, all types of L6C5 cultures showed a specific strong enhancement ($p < 0.01$) of caspase 3 activity; whereas in the C2C12 cells, a statistical increase was detectable in proliferating myoblasts ($p < 0.05$), but not in myotubes. Electrophoretic analysis showed $CoCl_2$-induced DNA fragmentation, which was present in the typical ladder form only in myotubes. As previously described for C2C12 cells [37], our results further confirmed that, in both C2C12 and L6C5 proliferating myoblasts, apoptosis did not involve DNA degradation into a typical ladder form (Figure 5A). Indeed, in L6C5 and

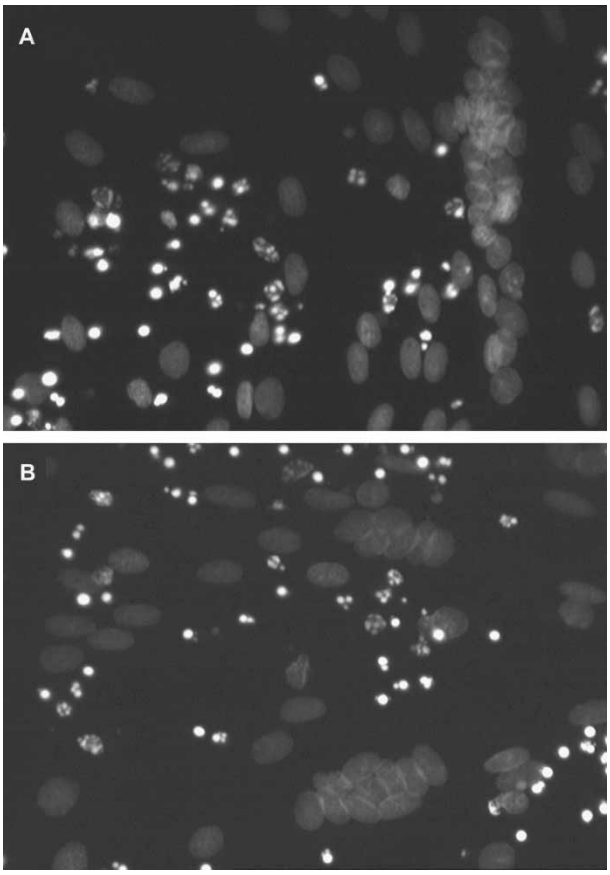

Figure 4. Apoptosis induced by $CoCl_2$ in differentiated cultures. Hoechst-stained differentiated cells from L6C5 culture treated with 500 μM $CoCl_2$ (A) or from C2C12 culture treated with 700 μM $CoCl_2$ (B).

C2C12 myoblasts, we did not observe the common 180–200 bp nucleosomal fragmentation; rather, we detected only the induction of high molecular weight (12.000–4.000 bp) DNA fragments specifically related to the $CoCl_2$ treatment. Despite the fact that the caspase 3 activation and apoptotic nuclei were

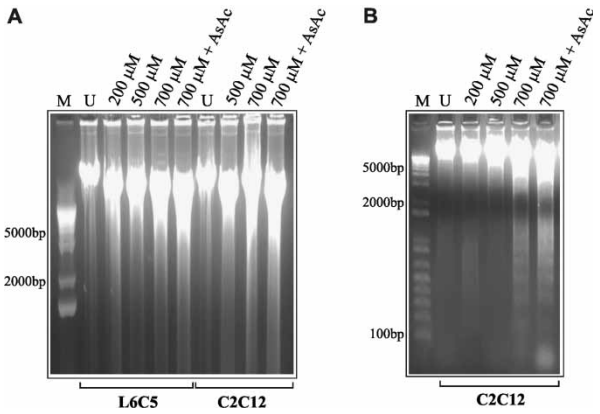

Figure 5. DNA laddering in skeletal muscle cells exposed to cytotoxic concentrations of $CoCl_2$. (A) L6C5 and C2C12 undifferentiated myoblasts. M represents DNA molecular weight marker, 1 kb DNA ladder. (B) L6C5 differentiated myotubes. U, untreated cells; M, DNA molecular weight marker, 1 kb DNA ladder.

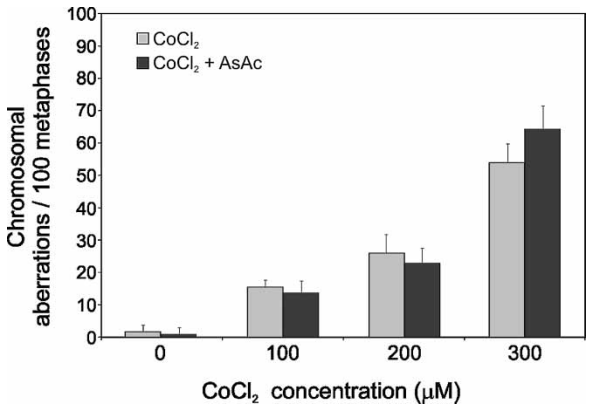

Figure 6. Effects of ascorbic acid 2-phosphate (100 μM) on the frequency of CA induced by pulse treatments with different concentrations of $CoCl_2$ in L6C5 proliferating myoblasts.

induced in both myoblast and myocyte cultures, the analysis of DNA laddering showed that $CoCl_2$ resulted in the typical internucleosomal DNA fragmentation in muscle cells only after culturing in differentiation medium (Figure 3B). As mentioned above for apoptotic nuclei frequencies, the extent of DNA fragmentation was also not reduced by AsAc supplementation in either cell line. These results suggested that neither the presence of the ROS scavenging activity, nor the AsAc capability to interfere with $CoCl_2$-induced HIF-1α stabilization, are able to modify cellular damage determined by this metal. To further confirm this hypothesis, we analyzed, in L6C5 myoblasts, the effect of AsAc on the $CoCl_2$-induction of CA, a nuclear damage already correlated to the *in vivo* and *in vitro* $CoCl_2$-genotoxicity [38]. All treatments with different concentrations of $CoCl_2$ for 1 h resulted in a statistical increase ($p < 0.05$) in the percentage of CA, which ranged from 15.5 ± 2.1 (100 μM $CoCl_2$) to 54.0 + 5.7 (300 μM $CoCl_2$), with no differences ($p > 0.05$) correlated to the supplementation in culture with AsAc (100 μM $CoCl_2$ + AsAc = 13.7 ± 3.6; 300 μM $CoCl_2$ + AsAc = 64.3 ± 7.1) (Figure 6).

Discussion

A decrease in oxygen level affects a variety of cellular processes and is also recognized as a major contributing factor in several pathologies [39,40]. In recent years, ROS have been suggested to be ideal messengers or mediators acting in the O_2-sensing system, and are involved in the signal transduction pathways by modulating gene activity in response to hypoxia [41]. In some situations, when sustained hypoxia would result in oxidative stress (i.e. an excess of free radicals overwhelming the antioxidant defense systems), ROS may be implicated in the induction of apoptosis and in hypoxia-related diseases [11,12]. The existence of a similar mechanism operative in skeletal muscle has been only poorly investigated to date. Some data suggest that during hypoxia, antioxidants can modulate pathways responsible for the preservation of skeletal muscle functions [42] and that, in the transition to low intracellular PO_2, a burst of intracellular ROS is formed [43]. However, the cellular responses involved in this process are unknown.

Since cobalt chloride can mimic the hypoxic response both in terms of gene up-regulation and cell death promotion in many cellular systems, we tested if $CoCl_2$ treatments in myogenic cells may represent a suitable *in vitro* model to explore the molecular and cellular response of skeletal muscle to hypoxia, as well as the potential involvement of free radicals in this response. We utilized L6C5 and C2C12 myogenic cell lines because, although closely related, they show differences in oxidative stress susceptibility [29].

In this work, we have demonstrated that in both L6C5 and C2C12 differentiated myotubes, non toxic $CoCl_2$ treatments triggered the activation of the HIF-1α, VEGF and, at least in C2C12, *iNOS* genes. In this respect, these *in vitro* experimental models of skeletal muscle completely support the *in vivo* data on the up-regulation of hypoxia-related genes [44] and confirm the involvement of iNOS as a downstream target gene in the signal transduction pathway activated by $CoCl_2$-simulated hypoxia [35]. We found no differences between L6C5 and C2C12 cells in gene activation, and the efficiencies of $CoCl_2$ exposure in regulating *iNOS* and/or *HIF-1α* and *VEGF* gene products were comparable in the two cell types. Although several lines of data suggest the involvement of ROS in gene modulation induced by $CoCl_2$ [24,33,44,45], this hypothesis was not fully verified in our *in vitro* models of skeletal muscle. Indeed, as reported in many different cell types [46,47], L6C5 and C2C12 skeletal myoblasts exposed to $CoCl_2$ also showed an increase in intracellular ROS. The degree of oxidant production was strictly dependent on both $CoCl_2$-concentration and the efficiency of endogenous antioxidant systems of the myogenic cell lines. However, the evidence that AsAc, but not trolox, can modulate $CoCl_2$-induced activation of the hypoxia-related genes in myogenic cells suggests that ROS are not specifically involved in HIF-1α stabilization and gene up-regulation. This has previously been demonstrated in other cell types. For example, Minchenko et al. [48] demonstrated in Hep-3B hepatoma cells that H_2O_2 is not an intermediary molecule involved in hypoxia or $CoCl_2$ sensing, whereas Salnikow et al. [25] concluded that ROS produced during exposure of cells to metals that mimic hypoxia were not involved in the activation of HIF-1α-dependent genes. Similarly, Griguer et al. [49], exploring the HIF-1α regulation in association with redox responsiveness to $CoCl_2$ treatment in glioma cells, found that ROS generated in D54-MG cells exposed to $CoCl_2$ did not participate in the hypoxic signal transduction pathways. The effect

of AsAc may be related to properties other than its scavenging activity. It is indeed known that the degradation of the HIF-1α protein depends on a family of prolyl hydroxylase enzymes [50], which require ascorbic acid as a cofactor: the co-exposure of cells to cobalt(II) and ascorbate reversed the metal-induced stabilization of HIF-1 α and HIF-1α dependent gene transcription [51].

When L6C5 and C2C12 undifferentiated or differentiated cells were treated with increasing concentrations of $CoCl_2$, a specific cytotoxic effect was detected. Similar to reports in alveolar macrophages [28] and in PC12 neuronal cells [52], we demonstrate here in skeletal muscle cells that $CoCl_2$-induced cell loss was correlated to caspase 3 activation and the execution of the apoptotic program. In both L6C5 and C2C12, the susceptibility of proliferating myoblasts to the induction of cytotoxicity and apoptosis by $CoCl_2$ was higher than in multinucleated myotubes, which are already known to become resistant to cell death through the upregulation of antiapoptotic genes and the inhibition of caspase-3 activity [53]. Recently, Vassilopoulos and Papazafiri [27] demonstrated that in HL-1 cardiomyocytes, ROS play a dominant role in the $CoCl_2$-induced apoptosis mediated via the mitochondrial pathway, since treatments with ascorbic acid, trolox, and other antioxidants partially reversed this effect. Although the sensitivity of C2C12 and L6C5 myoblasts to $CoCl_2$–induced cell death reflects their oxidant susceptibility, neither AsAc, already proven to protect L6C5 myoblasts from the apoptosis induced by H_2O_2 [29,30], nor trolox were effective against the cytotoxicity or apoptosis caused by $CoCl_2$. We thus concluded that oxidative stress was not the main mechanism involved in this process in skeletal muscle cells, and that the different susceptibilities of the two cell lines to apoptosis could reflect their differences in NFkB activity, already demonstrated by our group [29]. It is well recognized that this transcription factor may function more generally as a central regulator of stress responses, since NFkB activation, determined by different stressful conditions, including oxidative stress and exposure to chemicals, blocks apoptosis in several cell types by activating the transcription of many anti-apoptotic genes [54]. Thus, the higher sensitivity of L6C5 proliferating myoblasts to $CoCl_2$-induced apoptosis, compared to C2C12 myoblasts, as well as the reduction of susceptibility of L6C5 multinucleated myocytes after fusion, could be related to NFkB-regulated mechanisms other than redox homeostasis. The involvement of ROS-independent mechanisms in $CoCl_2$ induced apoptosis has been already described in PC12 neuronal cells [47] and in acute myeloid leukemic cell lines NB4 and U937 [55]. The mechanism by which $CoCl_2$ leads to apoptosis of skeletal muscle cells requires further investigation that is beyond the scope of this report, but the results from the cytogenetic analysis performed in this study allow some speculations. Since it has been recently reported that cobalt(II) acts as a topoisomerase II poison and that some of the genotoxic effects of cobalt are mediated through topoisomerase II [56], our finding that cytotoxic concentrations of $CoCl_2$ induce CA in L6C5 myoblasts, not modulated by AsAc, could suggest a role for DNA damage in apoptosis triggered by cobalt(II) in skeletal muscle cells. The involvement of DNA damage in apoptosis induced by hypoxia in skeletal muscle cells is not known, and further studies with myogenic cell lines will be necessary to validate this hypothesis and the subsequent utilization of $CoCl_2$ as a model for hypoxia-induced cell damage in skeletal muscle.

In conclusion, $CoCl_2$-simulated hypoxia in cultured myoblast cell lines may represent a useful *in vitro* model to study gene up-regulation and the signal transduction pathway switched-on by hypoxia in skeletal muscle, since exposure of both L6C5 and C2C12 cells to $CoCl_2$ resulted in up-regulation of HIF-1α-related genes, such as *VEGF* and *iNOS*. Although $CoCl_2$ stimulated the production of ROS in L6C5 and C2C12 myoblasts in proportion to the susceptibility of the specific cell line to oxidants, it is unlikely that ROS represent the main mechanism by which $CoCl_2$ affects muscle cells. Moreover, we have demonstrated that $CoCl_2$ is able to induce apoptosis in skeletal muscle cells, specifically with a high susceptibility of the undifferentiated myoblasts. This result added new insights on the toxic effect of $CoCl_2$ in mammalian cells, but the correlation of this phenomenon to the cellular damage induced by hypoxia requires further investigation.

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