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Original Contribution

Cadmium chloride-induced oxidative stress in skeletal muscle cells in vitro

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Abstract

The effects of cadmium chloride ($CdCl_2$) on oxidative stress in the skeletal muscle cell line C_2C_{12} were investigated. Myoblast cells that differentiated into myotubes were treated with $CdCl_2$ (1, 3, 5, 7.5, 10, and 12.5 μM) for 24, 48, and 72 h. Subsequent assay of cell homogenates for MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) reduction, neutral red uptake and nucleic acid content showed that cadmium was toxic to C_2C_{12} cells in a concentration-dependent manner. Glutathione-S-transferase activity (nmol μg of protein^{-1} min^{-1}) was increased with 1 and 3 μM $CdCl_2$ (36.9 ± 5.6 and 32.1 ± 6.0, respectively) compared to control cells (21.8 ± 1.5), but decreased at higher concentrations (7.5 μM = 15.9 ± 3.3, 10 μM = 15.9 ± 4.6, and 12.5 μM = 10.5 ± 2.8). An increase in malondialdehyde content (nmol μg of protein^{-1}), especially at high $CdCl_2$ concentrations (control = 7.3 ± 0.5; $CdCl_2$: 7.5 μM = 11.2 ± 3.1, 10 μM = 14.6 ± 3.8, and 12.5 μM = 20.5 ± 6.5) indicated that there was enhanced lipid peroxidation. Light and scanning electron microscopy showed that there was a concentration-dependent loss of adherent cells and the formation of vesicles indicative of cell death. These results indicated that $CdCl_2$ increased oxidative stress in C_2C_{12} cells, and this stress probably compromised cell adhesion and the cellular antioxidant defense mechanisms.

Keywords: Cadmium chloride; Myotubes; Oxidative stress; Skeletal muscle cells

Introduction

Cadmium (Cd) is an environmental and industrial pollutant with a wide variety of toxic manifestations, including lung fibrosis, kidney tubular dysfunction, hypertension, osteoporosis, and cancer [1–4]. Studies in animals have shown that exposure to Cd can lead to the formation of a variety of malignancies, including sarcomas [5], leukemia [6], and lung and prostate cancers [7]. Other studies have suggested a correlation between exposure to Cd and some types of human cancers [8], indicating that Cd can also promote carcinogenesis [9]. The promoter activity of Cd may involve oxidative stress, disruption of intercellular gap junction communication (IGJC) and alteration of the cytoskeleton [10–12]. Since Cd is generally a poor mutagen [13], the carcinogenic potential of this metal is unknown, but could contribute to nongenotoxic or indirectly genotoxic events that may enhance cell proliferation, depress apoptosis, and/or alter DNA repair [14]. Such injuries caused by Cd or other noxious agents probably lead to cell death [15]. Alternatively, Cd may act indirectly by attenuating cellular antioxidant defenses, thereby increasing the intracellular levels of hydrogen peroxide. The latter can in turn produce free radicals capable of breaking or crosslinking DNA or triggering lipid peroxidation. This indirect action of Cd may trigger a process associated with the formation of mutagenic adducts in DNA. Finally, Cd may interact with the metal-

Abbreviations: Cd, cadmium; $CdCl_2$, cadmium chloride; CDNB, 1-chloro-2,4- dinitrobenzene; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FCS, fetal calf serum; GST, glutathione *S*-transferase; HB, homogenization buffer; H_2O_2, hydrogen peroxide; HSPG, heparin sulfate proteoglycans; LPO, lipid peroxidation; MDA, malondialdehyde; MPO, *N*-methyl-2-phenylindole; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, nucleic acid content; NRU, neutral red uptake; PBS, phosphate-buffered saline; PBS-Ca^{2+}, phosphate-buffered saline calcium; ROS, reactive oxygen species.

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binding sites of proteins involved in DNA transcription, DNA replication, and DNA repair [15–17].

Although there have been marked advances in our understanding of how organic toxic agents can affect living organisms, the mechanisms by which toxic metals such as Cd produce their biochemical effects are still largely unknown [18,19]. The role of oxidative damage in the cytotoxicity, genotoxicity, and carcinogenicity of Cd has not been fully elucidated. The specific antioxidative response of tissues appears to be dependent not only on the nature of the reactive oxygen species (ROS), but also on the specific tissue and oxidative agent involved [17]. In muscle, for example, variations in the activities of antioxidant enzymes have been reported under different pathological conditions associated with free radical injury [20]. Differences in the mechanisms regulating antioxidant defenses in muscle may explain the phenotypic variability among muscle disorders in which ROS play a pathogenic role [21]. In this context, cellular metabolism, biosynthetic pathways, and cell adhesion molecules may be targets for metal toxicity [22–24]. Although the liver and kidney are specific target organs for the bioaccumulation of metals, Seidki et al. [25] have also reported high levels of Cd in skeletal muscle. Several investigations have examined the effects of Cd on skeletal and smooth muscle function [26–29]. Since Cd can induce lipid peroxidation, one of the main signs of oxidative damage and [24], and since oxidative stress is one of the main processes in a wide variety of muscle diseases and pathologies [20,30], as well as in protein wasting in skeletal muscle [31], in this work we examined the ability of $CdCl_2$ to alter the levels of oxidative stress in myotubes of cultured C_2C_{12} skeletal muscle cells.

Materials and methods

Cell culture

Myoblast C_2C_{12} cells were generously provided by Dr. Michael J. Tisdale (Laboratory of Cancer Research, Aston University, Birmingham, England). The cultures were grown in tissue culture flasks (Corning, NY) in DMEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Sigma), 1% penicillin, and 1% streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO_2. All of the experiments were initiated using cells grown to 90–100% confluence. To induce differentiation, the growth medium was replaced by medium supplemented with 2% horse serum. $CdCl_2$ (Sigma), prepared freshly for each experiment, was used at final concentrations of 1, 3, 5, 7.5, 10, and 12.5 μM and left in contact with the cells for 24 h.

Cytotoxicity assays

The viability of control and $CdCl_2$-treated C_2C_{12} myotubes was assessed based on MTT reduction, neutral red uptake (NRU) and nucleic acid content (NAC). The MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a sensitive, quantitative colorimetric assay that measures cell viability based on the ability of mitochondrial succinyl dehydrogenase in living cells to convert the yellow substrate MTT into a dark blue formazan product. For the assay, the medium containing $CdCl_2$ was removed and a solution containing 0.01% MTT was added to each well. After incubation for 10 min at 37°C, the medium was removed and the formazan solubilized in ethanol. The plate was shaken for 30 min and the absorbance was measured at 570 nm [32]. The NRU assay is a cell viability test based on the incorporation of dye into the lysosomes of viable cells following incubation with the test agents. After removal of the medium from the plates, a solution of 0.05% neutral red was added to each well followed by incubation for 3 h at 37°C. The cells were then washed with phosphate-buffered saline containing calcium (PBS-Ca^{2+}), followed by the addition of 1% glacial acetic acid and 50% ethanol to each well to fix the cells and extract the neutral red incorporated into the lysosomes. The plates were shaken for 20 min and the absorbance was measured at 540 nm [33]. For the NAC assay, monolayers of cells were solubilized with 0.5 N NaOH at 37°C for 1 h and the absorbance was measured at 260 nm; the results were expressed as a percentage of the control [34].

Analytical methods

After 24 h of treatment with $CdCl_2$, the cells were washed with cold PBS and collected in homogenization buffer (HB) (20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$, pH 7.2), and centrifuged at 10,000 rpm for 15 min at 4°C. Aliquots of homogenate supernatants were analyzed for glutathione-S-transferase (GST) activity based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the activity was expressed in nanomoles per microgram of protein per minute, using an extinction coefficient of 9.6, as described by Habig et al. [35]. The lipid peroxidation product malondialdehyde (MDA) was determined using MPO (*N*-methyl-2-phenylindole) as the substrate. The resulting absorbance was measured at 590 nm and the results were expressed in nanomoles per milligram protein [31]. The protein content was measured by the method of Lowry et al. [36].

Light (LM) and scanning electron (SEM) microscopy

Myotubes were cultured on coverslips and treated with various concentrations of $CdCl_2$ for 24 h prior to analysis by LM (Leica DMLM, Wetzlar, Germany). For SEM, other cells were fixed in 2.5% paraformaldehyde/glutaraldehyde (Sigma) in 0.1 M PBS, pH 7.4, and then washed in PBS followed by postfixation with 1% osmium tetroxide (Sigma) and dehydration in a graded ethanol series. The cells were then critical-point-dried (CPDO030–Balzers, BAL-TEC