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Cobalt induces oxidative stress in isolated liver mitochondria responsible for permeability transition and intrinsic apoptosis in hepatocyte primary cultures

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ABSTRACT

It is well established that cobalt mediates the occurrence of oxidative stress which contributes to cell toxicity and death. However, the mechanisms of these effects are not fully understood. This investigation aimed at establishing if cobalt acts as an inducer of mitochondrial-mediated apoptosis and at clarifying the mechanism of this process.

Cobalt, in the ionized species Co^{2+}, is able to induce the phenomenon of mitochondrial permeability transition (MPT) in rat liver mitochondria (RLM) with the opening of the transition pore. In fact, Co^{2+} induces mitochondrial swelling, which is prevented by cyclosporin A and other typical MPT inhibitors such as Ca^{2+} transport inhibitors and bongkrekic acid, as well as anti-oxidant agents. In parallel with mitochondrial swelling, Co^{2+} also induces the collapse of electrical membrane potential. However in this case, cyclosporine A and the other MPT inhibitors (except ruthenium red and EGTA) only partially prevent $\Delta\Psi$ drop, suggesting that Co^{2+} also has a proton leakage effect on the inner mitochondrial membrane. MPT induction is due to oxidative stress, as a result of generation by Co^{2+} of the highly damaging hydroxyl radical, with the oxidation of sulfhydryl groups, glutathione and pyridine nucleotides. Co^{2+} also induces the release of the pro-apoptotic factors, cytochrome *c* and AIF. Incubation of rat hepatocyte primary cultures with Co^{2+} results in apoptosis induction with caspase activation and increased level of expression of HIF-1α.

All these observations allow us to state that, in the presence of calcium, Co^{2+} is an inducer of apoptosis triggered by mitochondrial oxidative stress.

1. Introduction

Cobalt is an oligoelement present in almost all the animal and vegetal organisms; its biological importance is due to its essential role in the formation of vitamin B_{12} and other cobalamines. Vitamin B_{12} is necessary for the organism, because it is involved in the formation of some proteins and in the normal functionality of the nervous system. Its lack can cause pernicious anaemia and peripheral nervous system diseases (Karovic et al., 2006).

Cobalt is potentially toxic in the ionic form, Co^{2+}. Data in the literature indicate that cobalt is cytotoxic to many cell types, including neural cells (Wang et al., 2000) and can induce cell death by apoptosis and necrosis (Huk et al., 2004). It can cause DNA fragmentation (Zou et al., 2001), activation of caspases (Zou et al., 2002), increased production of reactive oxygen species (ROS) (Olivieri et al., 2001), augmented phosphorylation of mitogen-activated protein (MAP) kinases (Yang et al., 2004), and elevated levels of p53 (Chandel et al., 2000), as a consequence of the activation of hypoxia-inducible factor-1 (HIF-1) (Zou et al., 2001). In fact, in cultured cells, cobalt chloride mimics a hypoxic response. Like low oxygen tension, this metal is able to stabilize the α-subunit of HIF-1 (HIF-1α) by block-

Abbreviations: AdNT, adenine nucleotide translocase; AIF, apoptosis inducing factor; APF, 2-[6-(4′-amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid; BHT, butylhydroxytoluene; BKA, bongkrekic acid; DMF, dimethyl formamide; CsA, cyclosporin A; cyt *c*, cytochrome *c*; DTE, dithioerythritol; HIF-1, hypoxia-inducible factor-1; MPT, mitochondrial permeability transition; NAC, *N*-acetylcysteine; RLM, rat liver mitochondria; ROS, reactive oxygen species; RR, ruthenium red; TBARS, thiobarbituric acid-reactive species; $\Delta\Psi$, membrane potential.

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ing its ubiquitination and proteasomal degradation (Epstein et al., 2001; Morwenna and Ratcliffe, 1997). Increased levels of HIF-1α stimulate overexpression of a set of genes encoding several proteins such as heat shock proteins, which promote a physiological response linked to the recovery of cell homeostasis. In the same way the transcription of many pro-apoptotic factors, such as NIP-3 and NIX, is achieved, with the effect of leading to cell death (Bruick, 2000).

Many experiments have been performed on alveolar macrophages and PC12 cells (Zou et al., 2001; Tomaro et al., 1991). The way by which Co^{2+} is able to induce apoptosis still has to be discovered, but there is some evidence that it activates both the extrinsic and the intrinsic pathway. Zou et al. used a caspase 3-like inhibitor, which is able to inhibit programmed cell death partially, suggesting the peculiar role of this protein in the cobalt-mediated process (Zou et al., 2002). In spite of these observations, the molecular mechanism by means of which cobalt leads to cell death still has to be understood.

There is some evidence that it acts by activating the intrinsic apoptotic pathway, because its effect is blocked by caspase 9-inhibitors (Araya et al., 2002). This suggests that production of ROS induced by the metal acts directly on mitochondria to provoke the release of cytochrome *c* (cyt *c*) from external mitochondrial membrane, which leads to the activation of caspase 9 and to apoptosis (Pulido and Parrish, 2003). Similar conclusions have also been reported by other authors studying the toxic effects of cobalt in primary cultures of mouse astrocytes (Karovic et al., 2006). The interaction of Co^{2+} with mitochondrial function has been preliminarily investigated at the level of ATP synthesis, with inhibition of this phenomenon, probably ascribable to the opening of the transition pore (Bragadin et al., 2007).

The aim of our work is to explain the mechanism of cobalt-induced cell death and which is the role of mitochondria in this phenomenon. Our studies were performed on hepatocyte primary cultures and isolated liver mitochondria, because the highest quantities of physiological Co^{2+} in the body is contained in the liver, as in kidney, heart and spleen, whereas low concentrations are detected in serum, brain and pancreas (Derelank and Hollinger, 2002).

2. Materials and methods

2.1. Materials

Mouse monoclonal antibody anti-cyt *c* was purchased from Pharmingen, rabbit polyclonal antibody anti-apoptosis-inducing factor (AIF) was purchased from Chemicon International. Rabbit polyclonal antibody anti-caspase 3 and rabbit polyclonal antibody anti-HIF-1α were purchased from Santa Cruz Biothecnology. Fluorescence probe 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) was from Sigma. All other reagents were of the highest purity commercially available.

2.2. Mitochondrial isolation and standard incubation procedures

Rat liver mitochondria (RLM) isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM HEPES (pH 7.4), and 1 mM EGTA (Schneider and Hogeboom, 1950); EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard (Gornall et al., 1949). Mitochondria (1 mg protein/ml) incubated in a water-jacketed cell at 20 °C. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 50 μM Ca^{2+}, and 1.25 μM rotenone. Variations and/or other additions are given with each experiment.

The experiments were carried out at 20 °C in order to compare the results with those obtained in many other previous papers on the mitochondrial permeability transition (MPT) (e.g., see Gardini et al., 2001; DallaVia et al., 2006). Whole rat liver mitochondria exhibit a reversible broad gel to liquid crystalline phase transition at 0 °C (Blazyk and Steim, 1972) and at 20 °C the membrane is in the sol form. In MPT conditions, the fluidity of the membrane is greatly increased with respect to control conditions and increases still further as temperatures rise (Ricchelli et al., 1999). Therefore, the choice of 20 °C was made with the aim of minimizing alteration of the membrane during the MPT due to excessive fluidity. It should also be emphasized that, at higher temperatures, e.g., 30 °C, the respiratory chain operates at a high rate, producing anaerobiosis in the mitochondrial suspension within a few minutes, particularly in MPT conditions.

2.3. Determination of mitochondrial functions

Membrane potential ($\Delta\Psi$) was calculated on the basis of movement of the lipid-soluble cation tetraphenylphosphonium (TPP^+) through the inner membrane, measured using a TPP^+-specific electrode (Kamo et al., 1979). $\Delta\Psi$ determinations were corrected for non-specific intramitochondrial binding of TPP^+, as proposed by Jensen et al. (1986). Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control.

The protein sulfhydryl group oxidation assay was performed as in Santos et al. (1998). The redox level of glutathione was monitored as described in Tietze (1969). The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer with excitation at 354 nm and emission at 462 nm.

The production of H_2O_2 in mitochondria was measured fluorometrically by the Scopoletin method (Loschen et al., 1973) in an Aminco-Bowman 4-8202 spectrofluorometer.

Hydroxyl radical was detected fluorometrically by the probe APF with excitation at 490 nm and emission at 555 nm according to Setsukinai et al. (2003).

Lipid peroxidation was determined by monitoring the formation of thiobarbituric acid-reactive species (TBARS) according to Willis and Wilkinson (1966). TBARS were determined spectrofluorimetrically at 532 nm with an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Protein carbonyls were measured spectrophotometrically at 360 nm with the extinction coefficient of $22{,}000\ M^{-1}\ cm^{-1}$, according to Reznick and Packer (1994).

The cobalt ion content of the supernatant and its fluxes across the membrane were estimated by a centrifugal-filtration method (Toninello et al., 1985) with atomic absorption spectroscopy, on a Perkin-Elmer 110B spectrometer.

2.4. Detection of cyt c and AIF release

Mitochondria (1 mg protein/ml) were incubated for 15 min at 20 °C in standard medium with the appropriate additions. The reaction mixtures were then centrifuged at 13,000 × *g* for 10 min at 4 °C to obtain mitochondrial pellets. The supernatant fractions were further spun at 100,000 × *g* for 15 min at 4 °C to eliminate mitochondrial membrane fragments and concentrated five times by ultrafiltration through Centrikon 10 membranes (Amicon) at 4 °C. Aliquots of 10 μl of the concentrated supernatants were subjected to 15% SDS-PAGE for cyt *c* and 10% SDS-PAGE for AIF and analyzed by western blotting using mouse anti-cyt *c* antibody and rabbit anti-AIF antibody.