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MICROCALORIMETRY AS A TOOL FOR Cr(VI) TOXICITY EVALUATION OF HUMAN DERMAL FIBROBLASTS

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Occupational exposure to Cr(VI) causes various effects including deep skin ulcerations. Its action mechanisms are not fully understood. In the present study, the evaluation of human dermal fibroblasts heat production was monitored, using microcalorimetry. as part of Cr(VI) toxicity. In control cells, normal heat production was 15±5 pW/cell. Regardless of the Cr(VI) concentration tested (0 to 500 μM), heat production was inhibited over time periods ranging from 3 to 25 h. These results could be correlated with cell mortality and the IC_{50} for Cr(VI) was 29±4 μM. In the WST-1 bioassay, the IC_{50} was 35±5 μM (no statistical difference). Thus, Cr(VI) altered the metabolism of the fibroblasts, and led to cellular death. Microcalorimetry can be a useful tool for determining the toxic effect of suspect compounds implicated in the occurrence of pathologies.

Keywords: *cytotoxicity assay, fibroblasts, hexavalent chromium (Cr(VI), microcalorimetry*

Introduction

Occupational exposure to hexavalent chromium Cr(VI) is well known to be associated with bronchial asthma and lung cancer [1–4]. Cr(VI) has therefore been classified as carcinogenic to humans by the IARC (group1) [5]. Moreover, epidemiological studies have shown that Cr(VI) is responsible for deep skin ulceration and allergic dermatitis [6–8]. Thus, contact with Cr(VI) salts could induce Cr(VI) allergy. Despite recent progress in understanding the toxic potential of Cr(VI), the implicated mechanisms of Cr(VI)-induced cutaneous toxicity are not yet fully elucidated. In fact, few toxicological studies have examined skin damage after Cr(VI) exposure. Evidence in the literature suggests multiple possible targets of Cr(VI) toxicity. Cr(VI) may induce diverse responses in exposed cells and tissues. These different Cr(VI)-induced endpoints may depend on several mutually interfering factors such as concentration, the variety of intracellular targets, and the nature and/or extent of cellular damage [9, 10]. Therefore, as previously reported, it is important to establish the relationships between Cr(VI) exposure, specific cellular targets and resulting endpoints, to predict the type and extent of damage induced by this metal [11]. In the present study, we have evaluated the effects of Cr(VI) on the metabolism of human dermal fibroblasts. Fibroblasts were chosen because they are cells of dermal origin which provide durability and flexibility to the skin. These cells are considered to be a model system for investigating the epithelial toxicity of chromium [12].

The effect of Cr(VI) on the metabolism of these cells was assessed by microcalorimetry. This technique is a powerful tool that provides a continuous measurement of heat production. Thus useful information can be obtained on the activity of the overall metabolism of the cells qualitatively and quantitatively. Microcalorimetry is a direct non-invasive physical technique for measuring changes in thermodynamic parameters in biological systems. The technique monitors the metabolic processes at constant temperature. In this case, metabolism is considered as the sum of the energetic effects taking place in cells. Thus, the maximal thermal power and total heat production in calorimetric experiments can be used to evaluate the metabolic activity of cells.

Our results showed that fibroblast exposure to Cr(VI) caused the total inhibition of heat production and this inhibition was correlated with cell mortality. This dose-dependent phenomenon was compared with WST-1 viability measurements of cells assays, suggesting that mitochondria are one of the main intracellular targets of Cr(VI).

Experimental

Materials

Reagents

Potassium chromate (RP Normapur) was purchased from Prolabo (Fontenay s/Bois, France). Sodium dodecyl sulfate (SDS) was purchased from Sigma Aldrich (St. Quentin Fallavier, France). All chemicals

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for cell cultures were purchased from Eurobio (Les Ulis, France). WST-1 reagent was obtained from Roche Diagnostics (Meylan, France).

Normal human fibroblast culture

Normal human fibroblasts were isolated by the outgrowth method using infant foreskins obtained after circumcision [13]. The dermis was cut into small pieces of 0.5–1 mm^3 under sterile conditions. The small tissue pieces were seeded in culture dishes and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Fibroblasts were then cultured in complete DMEM without antibiotic at 37°C in a 5% CO_2 humidified atmosphere. A pure culture of fibroblasts was obtained within two weeks.

Methods

Microcalorimetric experiments

The fibroblasts were trypsinized (0.05% trypsin, 0.02% EDTA in PBS for 3–5 min at 37°C). After centrifugation, the cells were suspended in complete DMEM supplemented with 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. This suspension represented the sample for the microcalorimetric experiments. The total number of cells used in each measurement ranged from $2 \cdot 10^5$ to $2.5 \cdot 10^6$ cells.

The experiments were conducted in a heat-flow microcalorimeter (2277 Thermal Activity Monitor; Thermometric, Sweden) maintained at 37°C. Under sterile conditions, 900 μL of the cell suspension was placed in a 1.5 mL stainless steel vessel. A similar vessel containing the same volume of sterile DMEM served as reference. The vessels were transferred stepwise to the measurement position during 40 min as previously described [14]. The vessels were turbine-stirred at 60 rpm while heat production was measured continuously. The thermal power of the fibroblasts was determined by injection of an aliquot (100 μL) of 17.3 mM sodium dodecyl sulfate (SDS) solution into the calorimeter vessel containing the fibroblast suspension. The effect of Cr(VI) was studied similarly with a concentration range of 0–500 μM. For all compounds and concentrations, each experiment was independent and conducted at least three times. Data acquisition was carried out using Digitam 4.1 software (Thermometric, Sweden). The microcalorimeter was frequently dynamically calibrated in the course of the study. Power-time curves were thus automatically corrected and registered with the Tian equation used in Digitam 4.1.

Cytotoxicity assay (WST-1)

Fibroblasts were cultured in a 96-well culture plate for 24 h. Each well contained $2 \cdot 10^4$ cells. The cells were incubated with 0, 10, 20, 50, 100, 150, 200 and 250 μM of Cr(VI) in complete DMEM for 24 h at 37°C after which WST-1 assay was performed. The WST-1 assay was used to evaluate the effects of Cr(VI) on normal human fibroblast viability through the inhibition of metabolic activity [15]. The test is based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells to form a yellow-colored formazan dye, which is monitored by the determination of $OD_{450\ nm}$ using a microtiter plate reader (Multiskan RC, Labsystems, France). After Cr(VI) treatment, a second 30 min treatment with the tetrazolium salt WST-1 was performed and the $OD_{450\ nm}$ was measured.

Results and discussion

Figure 1 shows the thermal power curve obtained from fibroblasts treated with SDS. The cells produced a typical thermal power curve with a rapid rise followed by a plateau. At this equilibrium, SDS solution was injected, causing the production of a peak resulting from the exothermic dilution of the reagent. The SDS induced cell lysis with a subsequent decrease in thermal power to a value close to zero. The difference between the two levels ($\Delta(dQ/dt)$) corresponded to the fibroblast thermal power. Thermal power was determined for different cell populations (Fig. 2). Heat production was linearly correlated with the number of cells according to the equation: $dQ/dt = 15.1 \cdot$cell number $+0.1$ (r=0.975, P<0.001). For these experiments, the average power per cell was 15±5 pW/cell. This value is within the wide large range (5–134 pW/cell) reported for various cell types [16, 17]

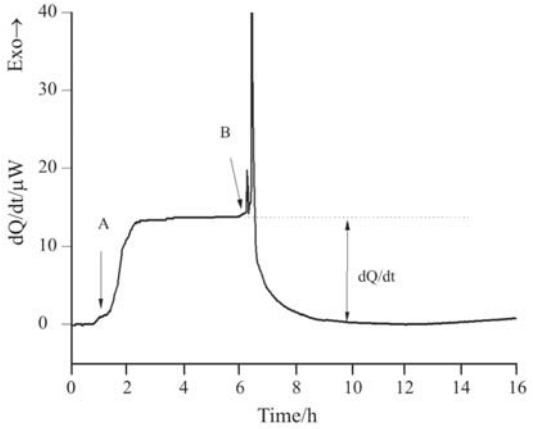

Fig. 1 Typical thermal power curve of fibroblasts treated with SDS. Arrow (A) indicates the introduction of the cell suspension to the measurement position; arrow (B), the injection of SDS (17.3 mM)

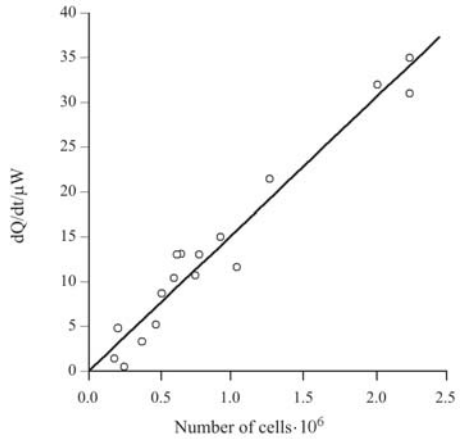

Fig. 2 Regression analysis between thermal power of fibroblasts and the number of the cells in suspension

and underlines the close relationship between thermal power and cell metabolism. As glucose was the only carbon source in the medium, one can assume that thermal power was associated with glucose assimilation [18–22] and considered as a quantitative parameter of cell metabolism. Figure 3 shows typical thermal power curves obtained from fibroblasts treated with Cr(VI). Without Cr(VI), the thermal power begins to decrease very slowly after 5 h to reach a steady state at around 30 h. However, the calculated power value was again 15 pw/cell. This occurrence is assumed to correspond to 'natural' cell death in a closed system. In fact, cell death cannot be due to cell lysis because, as observed in experiments carried out with SDS (Fig. 1), the thermal power should decrease faster. In addition, death cannot be due to lack of glucose nor low pH because complete DMEM medium used contains a large quantity of glucose (25 mM) and is buffered with bicarbonate (44 mM, pH 7.4). Thus in our experimental conditions, 'natural' cell death may be due to a lack of oxygen. By comparison, in the presence of Cr(VI) and for all concentrations, a steady state close to the baseline was reached, which we interpret as complete cell death caused by Cr(VI). As shown in Fig. 3, thermal power approached zero faster with increasing Cr(VI) concentration. In fact, after the addition of Cr(VI) the plateau was reached after around 20 and 4 h for 30 and 300 μM, respectively. Consequently, the lethal effect of Cr(VI) was dose dependent. To quantify this effect, the heat produced (Q) by the sample was determined by calculating the integral of the sample thermal power from the beginning of its decrease until it reached to a value close to zero. Figure 4 shows the relationship between heat production and Cr(VI) concentration. The value heat produced decreases with increasing Cr(VI) concentration. For our data, the best fitting curve was obtained for the model of Yerusalimsky [23] which we adapted according to the equation:

$$Q = Q_m \frac{K_I}{K_I + [Cr(VI)]} \quad (1)$$

where Q: heat produced (J), Q_m: maximum heat production (J), K_I: inhibition constant, expressed in concentration units, [Cr(VI)]: Cr(VI) concentration (μM).

For this model, when the Cr(VI) concentration is equal to the inhibition constant (K_I), the heat produced (Q) is equal to the half of the maximum heat production (Q_m) determined in the absence of Cr(VI). In order to express the effect of Cr(VI) in the form of toxicological data, the metal concentration leading to a 50% decrease in heat production was considered as IC_{50} so that $IC_{50}=K_I$. The calculated IC_{50} for Cr(VI) was thus 29±4 μM.

The effect of Cr(VI) on fibroblasts was also analysed using a conventional WST-1 viability test. The results are included in Fig. 5, clearly showing the relationship between cell viability and Cr(VI)

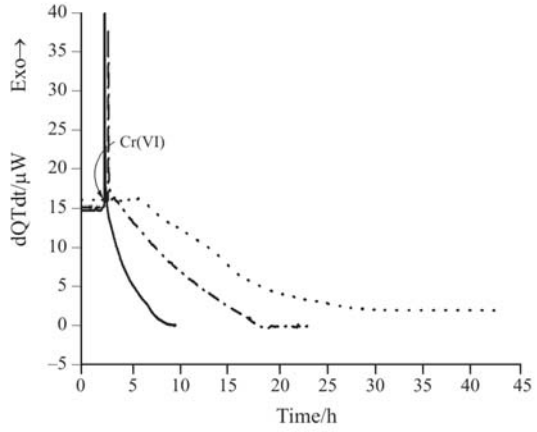

Fig. 3 Thermal power curves of fibroblasts treated with Cr(VI); · · · · · – 0; – · – · – – 30; — – 300 μM

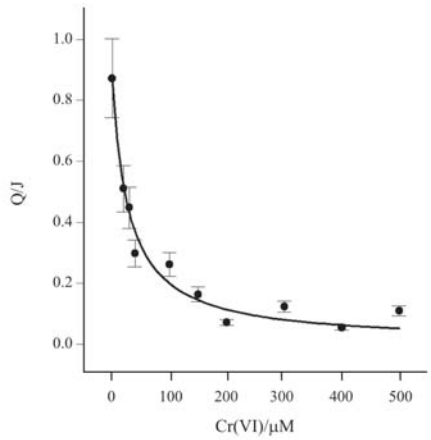

Fig. 4 Relation between the energy of the decrease of the thermal power in fibroblasts and concentration of Cr(VI). The values represent the mean ± SE of three independent experiments. The line corresponds to the regression curve obtained from the Yerusalimsky model as described in the text

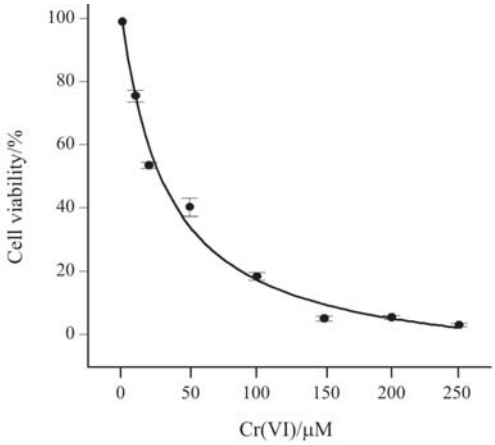

Fig. 5 Effect of Cr(VI) on fibroblast viability. The values represent the mean ± SE of 3 to 5 independent experiments. The line is the regression curve of all the data points using the model as described in the text

concentration. IC_{50} was calculated from the experimental data by fitting according to the equation derived from the classic Michaelis-Menten model:

$$I = 100 - \frac{I_m \times [Cr(VI)]}{IC_{50} + [Cr(VI)]} \quad (2)$$

where I: cell viability (%), I_m: maximum inhibition, IC_{50}: Cr(VI)concentration reducing cell viability on 50%, [Cr(VI)]: Cr(VI) concentration.

The calculated IC_{50} was 35±5 μM. The IC_{50} values obtained from microcalorimetry and cell viability experiments were not statistically different. The WST-1 test evaluated the mitochondrial activity of the cell and suggested that Cr(VI) modulated the cellular metabolism through the alteration of mitochondrial functions. This hypothesis is supported by a previous report showing that Cr(VI) induced mitochondria-dependent apoptosis [24]. This finding can also be connected to a recent study showing the effect of mercuric chloride on the metabolism of isolated mitochondria [25].

Conclusions

Microcalorimetry appears to be a convenient and easy technique for measuring metabolic processes through heat production in complex samples such as living cells. As opposed to standard bioassay procedures, this technique allows continuous measurements of the metabolism of living cells. We have thus shown that Cr(VI) impairs metabolic pathways of human fibroblasts and particularly glucose utilization. The thermogenesis measurements associated with cytotoxicity assays suggest that alteration of the mitochondria function is at least partly responsible for this disturbance.

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