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Effects of cobalt on haem proteins of erythropoietin-producing HepG2 cells in multicellular spheroid culture

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Abstract

The hypoxia-induced increase of spectrophotometrically measured light absorption at 560 nm, considered as reduced cytochrome *b*, in HepG2 cells is diminished after exposure to cobalt chloride (50 or 100 μM) for 18–36 h. The redox state of cytochrome *c* and cytochrome aa_3, however, remains stable, indicating a particular affinity of cytochrome *b* for cobalt. Erythropoietin production of HepG2 cells increases after application of cobalt chloride, whereas H_2O_2 production, as measured by the dihydrorhodamine technique, decreases. It is concluded that cobalt stimulates a signal cascade with cytochrome *b* as receptor and H_2O_2 as second messenger for regulating erythropoietin production.

Key words: HepG2 cell; Erythropoietin; Haem protein: H_2O_2; Cobalt; Oxygen sensing

1. Introduction

Erythropoietin (Epo), a 34 kDa glycoprotein that stimulates erythropoiesis, is synthesized under hypoxic conditions or during cobalt exposure in kidney and liver cells [5,8,11]. It has been shown that both stimuli induce two nuclear binding proteins with the essential binding site defined to be the −40 to −20 region upstream of the transcription initiation site in the human Epo gene [13]. For the liver a 29 bp element 3′ of the Epo gene has been identified to specifically bind a transacting factor that is active by hypoxia and cobalt [2,11]. The oxygen-sensing mechanisms underlying this hypoxia- and cobalt-mediated process have been intensively investigated at the molecular level in the hepatoma cell lines Hep3B and HepG2. The involvement of a specialized haem protein as an oxygen sensor has been suggested since CO inhibited hypoxia or cobalt induced Epo production [8]. Görlach et al. [7] identified in HepG2 cells a cytochrome *b* similar to the one of the NAD(P)H oxidase of neutrophils as an attractive candidate for this haem protein. It was proposed that H_2O_2 produced by this cytochrome acts as a second messenger to control the activity of the Epo gene.

The present paper investigates the influence of cobalt on the redox state of the different cytochromes as well as on the Epo and H_2O_2 production of HepG2 cells. The experiments have been carried out on HepG2 cells grown in multicellular spheroid culture as described by Görlach et al. [7].

2. Material and methods

2.1. Tissue culture and superfusion of the spheroids

HepG2 cells (ATCC HB 8065) were cultivated as spheroids as described by Görlach et al. [7] in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco, Eggenstein-Leopoldshafen, Germany) at 37°C in an incubator containing humid air with 5% CO_2 (Stericult 200; Labotect, Göttingen, Germany). Spheroid cultures were started with 2.5×10^5 cells in 130 ml medium in agarose-treated petri dishes (Gibco, Eggenstein-Leopoldshafen, Germany), and after reaching a diameter of 150 μm spheroids were further cultured in silicone-treated cylindrical spinner flasks (11 cm diameter × 24 cm high) with a spin rate of 40 rpm containing 250 ml of medium which was replenished twice a week. Experiments were carried out on HepG2 multicellular spheroids with a diameter between 700 and 800 μm containing about 60,000–70,000 cells per spheroid.

2.2. Erythropoietin determination

After incubation of HepG2 spheroids for 18 h under control conditions (PO_2: 140 Torr = 18.67kPa), hypoxia (PO_2: 10 Torr = 1.33 kPa) and $CoCl_2$ (50 or 100 μM; PO_2: 140 Torr) cell-free medium was collected by centrifugation at $150 \times g \times$ min. The collected medium was stored at −20°C. Epo was measured by radioimmunoassay in triplicate according to Fandrey et al. [4]. The assay was carried out using ^{125}I-labelled recombinant human Epo (rhu-Epo; 11–33 TBq/mmol; Amersham Buchler, Braunschweig, Germany) and antiserum from a rabbit immunized with rhu-Epo. The incubation mixtures each contained 100 μl of cell free medium, ^{125}I-labelled rhu-Epo and antiserum (1:2000). Calibration curves with human urinary Epo standard ranged from 2.5 to 250 mU/ml. Epo samples were incubated with the antibody for 48 h at 4°C prior to incubation with ^{125}I-labelled rhu-Epo for another 24 h. Then 1 ml of 16% polyethylene glycol (PEG 6000, Merck, Darmstadt, Germany) was added. The radioactivity was measured using a γ-counter (Beckman Instruments Inc., Fullerton, CA, USA). A log-logit plot was applied to calculate the Epo concentration of the samples from the calibration curve. The rate of Epo production was related to the protein content of the spheroids. The lower detection limit of the assay was determined to be 5 mU/ml.

2.3. Photometry

Spheroids were located in a superfusion chamber on a small bench with little holes of the same diameter as the spheroids. The superfusion chamber was described in detail elsewhere [3], therefore only a brief description is given below. Isotonic salt solution (Locke's solution) containing glucose (5 mM) was equilibrated with different O_2/CO_2

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mixtures in order to adjust the oxygen tension to various levels at pH 7.42. The flow rate through the chamber was 10 ml per min. Spheroids were supplied symmetrically with nutrients by this procedure. The temperature was maintained at 36°C. Oxygen tension, pH and temperature in the Locke's solution were continuously controlled. The superfusion chamber was mounted on the stage of a light microscope (Olympus, Hamburg, Germany) for light absorption measurements. Light from a halogen lamp (12 V, 100 W) transilluminated the spheroid only, since the bench was made opaque by sputtering with gold to avoid uncharacteristic light scattering. Light from the condensor was guided by the spheroid tissue to the objective and recorded by a photodiode-array spectrophotometer (MCS 210, Zeiss, Köln, Germany) connected to the third ocular of the microscope trinocular head via a light guide [3].

2.4. Measurement of H_2O_2 production

Following Cross et al. [3] dihydrorhodamine 123 (Molecular Probes, Eugene, OR, USA) was dissolved in dimethylsulphoxide to give stock solutions of 50 mM and stored under N_2. The non-fluorescent dihydrorhodamine is converted to fluorescent rhodamine in the reaction with H_2O_2 inside HepG2 cells. Images of the fluorescent HepG2 cells were obtained using a Bio-Rad MRC 600 confocal scanning microscope (Bio Rad, München, Germany) mounted on a Zeiss IM405 inverted microscope (Zeiss, Köln, Germany). The spheroids were placed on a bench as described above, in the superfusion chamber which was mounted on the stage of the inverted microscope. For comparative measurements of spheroids under control and $CoCl_2$ conditions, pin hole diameter, black level and gain of the confocal scanning microscope were kept constant.

2.5. Statistical analysis

Statistical differences were assessed using Student's *t*-test. Differences were considered to be significant at $P < 0.05$. Values are given as means ± S.D.

3. Results and discussion

3.1 Epo production

Epo production in spheroids under control conditions with a PO_2 of 140 Torr (18.67 kPa) in the culture medium was 15.6 ± 6.1 U/100 mg of protein ($n = 7$). After 18 h incubation under hypoxic conditions with a PO_2 below 10 Torr (1.33 kPa) in the culture medium Epo production was significantly enhanced to 51,2 ± 36.2 U/100 mg of protein ($n = 4$; $P < 0.0267$). Exposure to 50 μM or 100 μM $CoCl_2$ for 18 h under normoxic conditions (PO_2 = 140 Torr = 18,67 kPa) resulted into an increased Epo production of 28.7 ± 10.6 U/100mg of protein ($n = 4$; $P < 0.0263$) or 21.7 ± 9.1 U/100mg of protein ($n = 3$; $P < 0.238$) respectively. Each experiment comprises the Epo production rate of about 150 spheroids.

3.2. Haem proteins

To investigate the influence of cobalt on the redox state of cytochromes in HepG2 spheroids hypoxia (PO_2 = 3 Torr = 0.399 kPa) minus aerobic steady-state (PO_2 = 140 Torr = 18.67 kPa) difference spectra have been recorded in single spheroids under control conditions or after incubation with different concentrations of $CoCl_2$. The recorded difference spectra were normalized to the highest absorption consistently seen at 552 nm corresponding to the absorption maximum of cytochrome *c*. As shown in Fig. 1 (upper panel) normalized difference spectra obtained from HepG2 spheroids incubated with 100 μM $CoCl_2$ (broken line) revealed a significant deviation from the spectra obtained from control spheroids (solid line) at 560 nm. By subtracting the normalized $CoCl_2$ spectra from normalized control spectra the maximal deviation at 560 nm was calculated as 24%, as shown in the lower panel of Fig. 1. A calculated difference of 16% could be measured in spheroids treated with 50 μM $CoCl_2$ for 18 h ($n = 2$) and of 13% in spheroids treated with 50 M for 36 h ($n = 3$) without a changed redox state of cytochrome *c* and aa_3 (data not shown). These results suggest that pretreatment with $CoCl_2$ leads to specific reduction of a *b*-type cytochrome in HepG2 cells, resulting in decreased absorption changes after acute exposure to hypoxia, whereas the redox state of the other cytochromes is not affected by cobalt chloride. The similar degree of cytochrome *b* reduction after 18 and 36 h, as well as the clear dose dependence, further underlines the specific reaction of cytochrome *b* with cobalt.

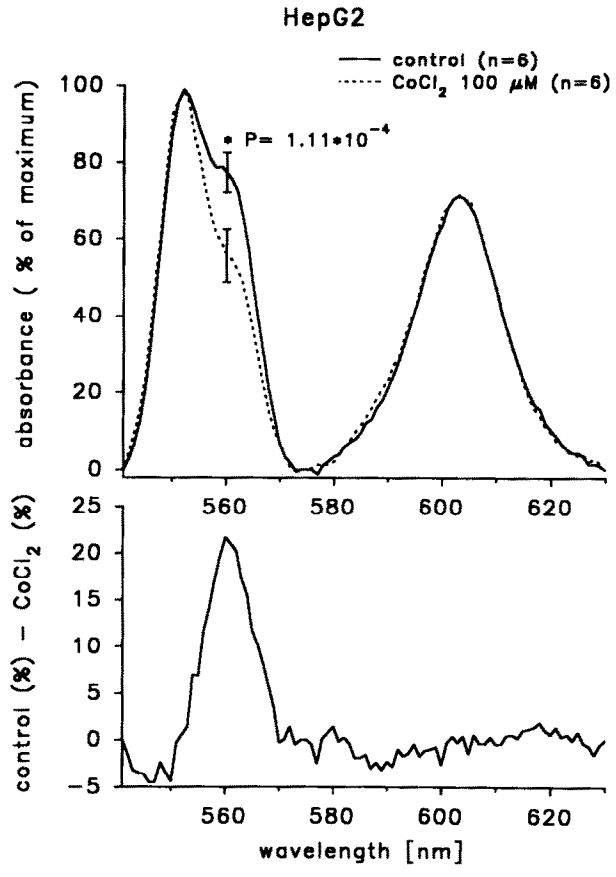

Fig. 1. Normalized hypoxia minus aerobic steady-state light absorption difference spectra of HepG2 spheroids (upper panel) of control spheroids and spheroids treated with 100 μM $CoCl_2$ for 18 h. Mean curve of six spheroids under control conditions (solid line) shows typical peaks at 552 nm (cytochrome *c*) and 603 nm (cytochrome aa_3) as well as a broad shoulder between 558 nm and 564 nm (cytochrome *b*). Mean curve of six spheroids treated with cobalt (dotted line) demonstrates a significant deviation peaking at 560 nm as shown with the calculated difference of both mean curves (lower panel). The level of significance (*P*) and the number of spheroids (*n*) can be seen.

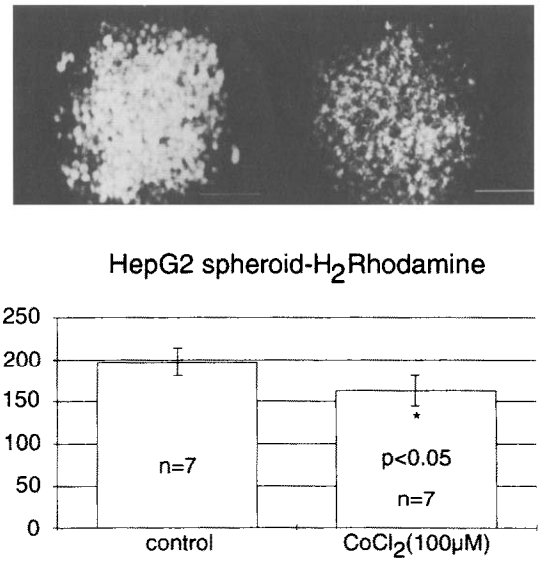

Fig. 2. Optical imaging of H_2O_2 in HepG2 cells at the surface of multicellular spheroids under control conditions (left side) and after 18 h treatment with 100 μM $CoCl_2$ (right side). Fluorescence intensity is shown with white as highest intensity and black as lowest intensity. Bars = 100 μm (upper panel). Mean value and standard deviation of rhodamine 123 fluorescence in arbitrary units of spheroids under control and $CoCl_2$ conditions. n = number of spheroids.

3.3. H_2O_2 production

The confocal microscope scans and collects emitted light only from within the plane of focus of the objective lens [3]. The image obtained is the fluorescence from a discrete slice of about 10 μm. Non-fluorescent cells are not visible. Fig. 2 (upper panel) shows optical imaging of HepG2 cells at the surface of multicellular spheroids under control conditions (left side) and after incubation with 100 μM $CoCl_2$ for 18 h (right side). The generation of fluorescent rhodamine 123 suggests that HepG2 cells under control conditions produce more H_2O_2 than after treatment with cobalt. Accordingly, mean fluorescence intensity (Fig. 2, lower panel) was significantly higher under control conditions with 197.29 ± 14.31 ($n = 7$, $P < 0.0016$) than after cobalt treatment with 162.10 ± 17.88 ($n = 7$). This effect was not measurable after incubation with 50 μM $CoCl_2$ for 18 h.

3.4. Conclusions

The signal transduction pathway for oxygen sensing in HepG2 and Hep3B cells from the putative sensor protein after interaction with hypoxia or cobalt to an enhanced Epo production involves a complicated signal cascade including protein kinases [9]. The *cis* elements and trans-activating factors regulating Epo gene expression have been partly identified, and the binding of an hypoxia-inducible factor to an hypoxia-responsive enhancer has been described [2,12]. This signal transduction pathway seems to be a widespread biological phenomenon as Maxwell et al. [10] reported that transient transfection of different non-Epo-producing cell lines with a plasmid containing the Epo hypoxia-responsive enhancer attached to a reporter gene results in hypoxia-induced up-regulation. The involvement of a neutrophil type cytochrome *b* as an oxygen-sensing protein has been suggested beside HepG2 cells in the carotid body (for review see [1]) and in neuroepithelial bodies of the lung [14]. Our experiments indicate that this cytochrome *b* has a particular affinity for cobalt. It remains to be elucidated in future experiments in what way the induced reduction of cytochrome *b* is linked to the declined level of H_2O_2, which has been shown to attenuate the hypoxia-induced Epo production in HepG2 cells [6], and whether these parameter changes are connected to variations in Epo production.

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