The influence of phenobarbital on cytochromes and reactive oxygen species in erythropoietin producing HepG2 cells

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Abstract Light absorption photometry of HepG2 cells treated with phenobarbital for enhancing the content of cytochrome P-450 and the synthesis of erythropoietin revealed an influence on all cytochromes detectable in the wavelength range between 400 and 620 nm. No correlation was found between specific changes of cytochrome P-450 absorption and increased EPO synthesis as proposed earlier by Fandrey et al. (Life Sci. (1990) 47, 127–134). In the present study, however, the increased erythropoietin synthesis could be related to a decreased intracellular hydroxyl radical level described as crucial for the oxygen regulated gene expression (Kietzmann et al., Biochem. J. (1998) 335, 425–432; Porwol et al., Eur. J. Biochem. (1998) 256, 16–23).

Key words: HepG2 cell; Erythropoietin; Oxygen sensing; Phenobarbital; Cytochrome P-450; Reactive oxygen species

1. Introduction

One possible signal cascade leading to oxygen dependent erythropoietin (EPO) gene expression [2] might involve a cytochrome [4] which produces H_2O_2 as a function of the oxygen partial pressure (PO_2) [5]. H_2O_2 may be degraded to hydroxyl radical ($^{\bullet}OH$) by a perinuclear Fenton reaction [6] which influences transcription factors regulating EPO gene expression [7]. As the oxygen sensing cytochrome Görlach et al. [4,8] and Ehleben et al. [6] proposed a low output NADPH oxidase. Similarities in light absorption spectra, subunit composition and the capacity for reactive oxygen species (ROS) production makes it likely that the oxygen sensor cytochrome is an isoenzyme of the high output NADPH oxidase of neutrophils. Fandrey et al. [1], however, proposed that the cytochrome P-450 monooxygenase might be a possible candidate for the oxygen sensing protein in HepG2 cells. The activity of cytochrome P-450 requires NADPH and molecular oxygen with a half-saturation at a PO_2 of 20–100 Torr [9]. HepG2 cells exposed to phenobarbital to enhance the cytochrome P-450 content, responded with an increased EPO synthesis. EPO synthesis was blunted by diethyldithiocarbamate probably by inhibiting the cytochrome P-450 reductase. Cytochrome P-450 has been proposed as microvascular oxygen sensor as well due to the regulatory function of various cytochrome P-450 metabolites of arachidonic acid influencing vascular tone primarily by affecting K^+ channel activity [10].

The question was therefore addressed whether light absorption spectral analysis as described by Ehleben et al. [6] and Porwol et al. [3] is able to detect specific upregulation of the cytochrome P-450 system in HepG2 cells exposed to phenobarbital. Furthermore the intracellular level of $^{\bullet}OH$ was determined to see whether enhanced EPO synthesis after phenobarbital treatment was due to decreased levels of the radical.

We found that cytochromes other than cytochrome P-450 showed higher absorption changes after exposure to phenobarbital for 72 h. This may indicate either an enhanced cytochrome synthesis and/or changes in the redox state. Because no obvious change in absorption of a single cytochrome was evident the identification of the primary oxygen sensor cytochrome by spectrophotometrical analysis after phenobarbital application appears to be impossible. However, we found significantly decreased intracellular $^{\bullet}OH$ level after 72 h phenobarbital exposure which were accompanied by enhanced EPO production. Since the control of intracellular $^{\bullet}OH$ levels has been shown to be critical for EPO gene expression [7] the reduced level of the $^{\bullet}OH$ radical may explain increased EPO production.

2. Materials and methods

2.1. Tissue culture

HepG2 cells (ATCC HB 8065) were cultivated in monolayer and spheroid tissue culture as described by Görlach et al. [4] in RPMI 1640 medium (Life Technologies, Eggenstein-Leopoldshafen, Germany) supplemented with 2 mM L-glutamine, 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in an incubator containing 5% CO_2 in humidified air (Stericult 200, Labotect, Göttingen, Germany). Spheroid cultures were started with 2.5×10^5 cells in agarose treated petri dishes (Life Technologies) with 130 ml medium. After reaching a diameter of 150 μm spheroids were transferred into siliconized cylindrical spinner flasks (11 cm diameter and 24 cm high). Spin rate was set at 40 rpm of the 250 ml medium which was replenished twice a week. For phenobarbital (Merck, Darmstadt, Germany) treatment the drug was added to the spheroids (diameter between 700 and 800 μm) kept in petri dishes at a concentration of 1.5 mM 72 h prior to the experiments. Control spheroids were kept in petri dishes for the same time. During the absorption measurements the solutions used contained no phenobarbital [1].

2.2. Light absorption photometry

Spheroids were located in a superfusion chamber on a small opaque bench with little holes of the same diameter as the spheroids. The superfusion chamber has been described in detail before [4,8]. Briefly isotonic salt solution (Locke's solution) containing glucose (5 mM) was equilibrated with different O_2/CO_2 mixtures in order to adjust the desired PO_2 at pH 7.42. The flow rate through the chamber was 30 ml per minute. 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 controlled by corresponding probes. For light absorption measurements, the superfusion chamber was mounted on the stage of a light microscope (Olympus, Hamburg, Germany). White light from a halogen bulb (12 V, 100 W) transilluminating the spheroid only passed to the objective (40×). Absorption changes at different wavelengths were recorded by a photodiode-array spectrophotometer (MCS 210, Zeiss, Cologne,

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Germany) connected to the third ocular of the microscope trinocular head via a light guide. Difference spectra were calculated by automatically subtracting the absorbance recorded under conditions of 20% O_2, 3% CO_2 and 77% N_2 (aerobic steady state) from the spectra under conditions of 3% CO_2 and 97% N_2 or 97% CO (N_2/CO steady state). Difference spectra were subsequently analysed and plotted using the software package TechPlot (Dr. Dittrich, Braunschweig, Germany).

2.3. Reactive oxygen species

For measuring intracellular ROS levels after three days of phenobarbital (1.5 mM) treatment HepG2 cells cultured in 96 multi-well plates were stained with dihydrorhodamine 123 (25 μM; Molecular Probes, Eugene, OR, USA) for half an hour [4]. Dihydrorhodamine 123 is a non-fluorescent dye described to specifically detect H_2O_2 (Molecular Probes). The reaction with H_2O_2, however, is rather slow under ambient conditions. It has been shown by Ehleben et al. [6] and Porwol et al. [3] that the intracellular conversion of non-fluorescent dihydrorhodamine 123 to fluorescent rhodamine 123 in the presence of H_2O_2 is due to a reaction with $^{\bullet}OH$ generated by a perinuclearly located iron mediated Fenton reaction. Thirty minutes after carefully washing each well with PBS intracellular rhodamine 123 fluorescence intensity was measured using a multi-well scanning fluorescence photometer (Titertek, Fluoreskan II, Flow, Meckenheim, Germany). Forty-eight wells were recorded in each experiment.

2.4. Metabolic activity

Metabolic activity was determined by the commercially available MTT assay (Cell proliferation Kit I (MTT), Boehringer Mannheim, Germany). The assay is based on the cleavage of the tetrazolium salt MTT, in the presence of an electron coupling reagent, i.e. succinate tetrazolium reduction by active mitochondria. HepG2 cells were cultured in the presence of phenobarbital (1.5 mM) for three days in a 96-well tissue culture plate (Falcon) at 37°C at a starting cell density of 0.5×10^5 cells per well. Cells were then incubated with the MTT solution for 4 h when the water-insoluble formazan dye is formed. After solubilization, the formazan dye was quantitated by measuring absorbance at 540 nm using a scanning multi-well spectrophotometer (MR5000, Dynatech, Denkendorf, Germany).

2.5. Erythropoietin mRNA measurements

HepG2 spheroids treated with phenobarbital for three days were washed with PBS after removal of the spent cell culture medium and lysed with 700 μl of guanidinium thiocyanate (4 M with 0.1 M β-mercaptoethanol). Total RNA was extracted by the acid-phenol method [11]. After dissolving the RNA in diethylpyrocarbonate treated water the concentration was determined by measuring the absorbance at 260 nm. One μg of total RNA was reversed transcribed into first strand cDNA using oligo-dT_{15} as primer for the reverse transcriptase M-MLV RT-superscript (Gibco Life Technologies) in a total volume of 25 μl. Reverse transcription was performed at 42°C for 60 min after an initial denaturation step at 68°C for 10 min. The reaction was terminated by boiling the samples for 10 min. Until quantitation by a competitive PCR cDNA stocks were kept at −20°C. Competitive PCR for EPO mRNA was performed exactly as described [12]. Results of the quantitative PCR are expressed in amol/μg of total RNA.

2.6. Statistical analysis

Data are presented as mean ± standard deviations. For statistical analysis the Student's *t*-test was performed. Differences were considered significant when the *P*-value was <0.05.

3. Results

3.1. Light absorption spectra

Fig. 1a shows the mean value of N_2 minus aerobic steady state difference spectra (dotted line) of 9 single untreated HepG2 spheroids. In the wavelength range between 400 nm and 620 nm characteristic α, β and γ absorbance peaks of the

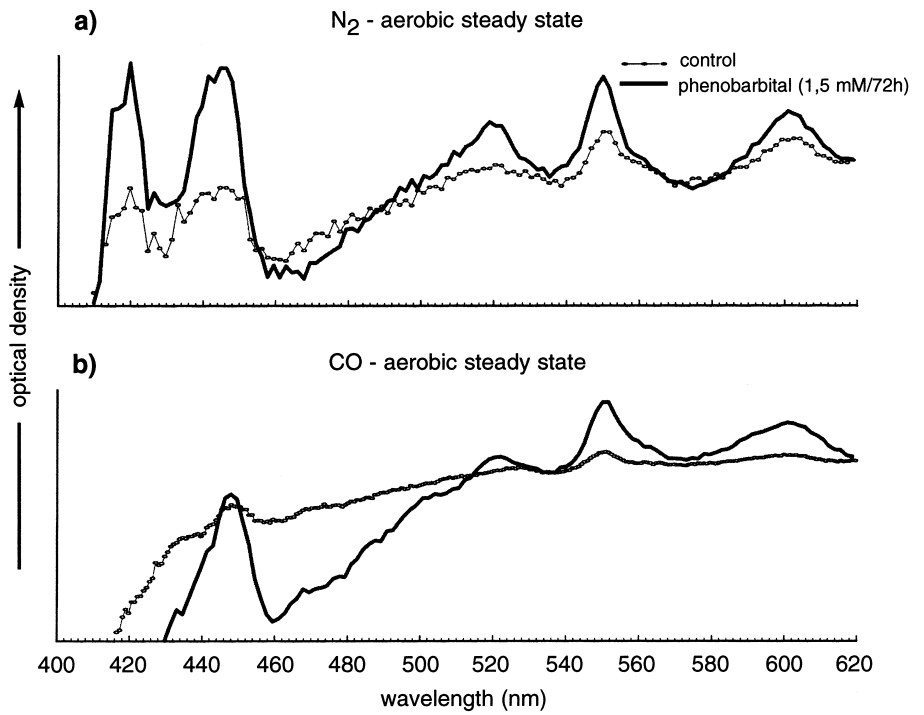

Fig. 1. Light absorption photometry (*X*-axis = wavelength in nm, *Y*-axis = optical density in arbitrary units). a: Dotted line: mean value of 9 N_2 minus aerobic steady state light absorption difference spectra recorded in the wavelength range between 400 nm and 620 nm on HepG2 spheroids under control conditions. Full line: mean value of 9 N_2 minus aerobic steady state light absorption difference spectra recorded in the same wavelength range on HepG2 spheroids after treatment with 1.5 mM phenobarbital for 72 h. b: Dotted line: mean value of 9 CO minus aerobic steady state light absorption difference spectra recorded in the wavelength range between 400 nm and 620 nm on HepG2 spheroids under control conditions. Full line: mean value of 9 CO minus aerobic steady state light absorption difference spectra recorded in the wavelength range between 400 nm and 620 nm on HepG2 spheroids after treatment with 1.5 mM phenobarbital for 72 h.

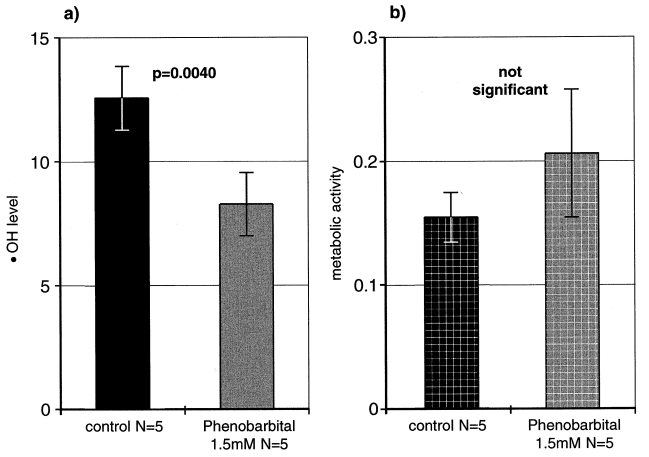

Fig. 2. Hydroxyl radical generation and metabolic activity (*Y*-axis = arbitrary units, *P* = significance level). a: Intracellular •OH level of HepG2 cells under control conditions and after treatment with 1.5 mM phenobarbital for 72 h. b: Metabolic activity (MTT-test) of HepG2 cells under control conditions and after treatment with 1.5 mM phenobarbital for 72 h.

different cytochromes contributing to the spectrum can be identified: peaks are for cytochrome *c* at 550, 520 and 420 nm, cytochrome aa_3 at 602, 520 and 445 nm or cytochrome *b* at 560, 530 and 430 nm [13,14]. Deconvolution of these difference spectra revealed – beside the mitochondrial cytochromes – a non-mitochondrial cytochrome b_{558} peaking at 558 nm [3,6]. Furthermore, the broad shoulder between 440 and 455 nm suggests the contribution of cytochrome P-450. The full line in Fig. 1a represents the mean value of N_2 minus aerobic steady state difference spectra of 9 single HepG2 spheroids exposed to 1.5 mM phenobarbital for 72 h. Light absorbance at all characteristic cytochrome peaks as described above was about doubled compared to control conditions without a significant shift in the peaks of the absorption maxima with respect to the wavelength. No specific single cytochrome could be identified as being specifically affected by phenobarbital treatment.

Fig. 1b shows the mean value of CO minus aerobic steady state difference spectra of the 9 control HepG2 spheroids (dotted line) as well as of the same 9 HepG2 spheroids treated with 1.5 mM phenobarbital for 72 h. Due to the specific CO affinity of cytochrome P-450 [15] the characteristic light absorption peak at 450 nm is prominent particularly after phenobarbital treatment (full line). The anoxic situation of the spheroid tissue due to superfusion with 97% CO-equilibrated medium leads to a reduction of the mitochondrial and non-mitochondrial cytochromes (see characteristic peaks at 520, 550 and 602 nm and the shoulder at 560 nm).

3.2. ROS formation and metabolic activity

Phenobarbital treatment caused a significant decrease of the intracellular •OH level in HepG2 cells when measured with the dihydrorhodamine 123 technique (Fig. 2a). Metabolic activity, determined with the MTT assay, increased in phenobarbital treated HepG2 cells (Fig. 2b). A higher mitochondrial mass or a change in the mitochondrial redox state as one may assume from the light absorption measurements (Fig. 1) could be responsible for this effect. The higher metabolic activity excludes toxic effects of phenobarbital on HepG2 cells.

3.3. EPO production

HepG2 spheroids with a diameter between 700 and 800 μm were treated in 2 experiments with 1.5 mM phenobarbital for 72 h. Each experiment includes about 20–30 spheroids. EPO mRNA increases from 116 amol/μg total RNA under control conditions to 174 amol/μg total RNA under phenobarbital treatment. These results indicate that HepG2 cells in multicellular spheroid culture as they were used for photometric studies respond to phenobarbital treatment with an increased EPO production as described by Fandrey et al. [1] for HepG2 cells in monolayer culture.

4. Discussion

Our experiments confirm earlier results [1] that phenobarbital enhances hypoxia-induced EPO synthesis in HepG2 cells. CO versus aerobic steady state difference spectra revealed an enhanced absorption peak at 450 nm most probably caused by an increased content of the Fe^{2+} form of cytochrome P-450 due to phenobarbital exposure [15]. Interestingly, phenobarbital also induced higher absorption peaks at wavelengths typical for mitochondrial and non-mitochondrial cytochromes. In line with these findings is the enhanced succinate tetrazolium reduction by mitochondria as found in the MTT assay. However, our spectral analysis does not allow to distinguish whether the concentration or the redox state, i.e. the activity state of the mitochondria were changed by phenobarbital treatment. This general influence of phenobarbital on cytochromes in HepG2 cells impedes the identification of a single heme protein as part of the primary oxygen sensor.

Different mitochondrial and non-mitochondrial proteins have been proposed as potential cellular oxygen sensors. The rat mitochondrial outer membrane-bound benzodiazepine receptor (MBR) is involved in steroidogenesis, heme biosynthesis, cell growth and differentiation, control of mitochondrial respiration, and immune and stress response. When MBR was expressed in wild-type and TspO(−) (tryptophan-rich sensory protein) strains of the facultative photoheterotroph bacterium *Rhodobacter sphaeroides*, MBR substitutes for TspO and inhibits – in response to oxygen – the expression of genes necessary for photosynthesis. Thus MBR was proposed to act as a mitochondrial oxygen sensor [16]. In isolated perfused cat carotid bodies CO competed with O_2 in oxygen sensing. The action spectrum of the photochemical response was characteristic of the CO bound to mitochondrial cytochrome a_3 suggesting that this cytochrome acts as a possible oxygen sensor [17]. Poisoning the respiratory chain, however, does not affect the O_2-dependent activation of hypoxia inducible factor 1 (HIF-1), the principal transcription factor complex in control of the hypoxia induced expression of the genes encoding erythropoietin, glycolytic enzymes and vascular endothelial growth factor [18]. Therefore, it is likely that a non-mitochondrial heme proteins acts as an oxygen sensor. A candidate is a low output NAD(P)H oxidase closely related to or even an isoenzyme of the respiratory burst (high output) NADPH oxidase of neutrophils [4,8]. This low output NAD(P)H oxidase was proposed to be responsible for the oxygen-dependent generation of H_2O_2, the putative intracellular messenger between the sensor and the transcriptional apparatus [5]. Components of such an oxidase could be localized immunohistochemically in the cell membrane of HepG2 cells [6]. This notion is supported by the effect of diphenylene

iodonium (DPI), a typical inhibitor of the NADPH oxidase, tested on five genes which show oxygen-regulated expression: erythropoietin, vascular endothelial growth factor, lactate dehydrogenase-A, glucose transporter-1 and placental growth factor. For each gene, the response to hypoxia was specifically inhibited by low doses of DPI [19]. However, DPI inhibition of the NADPH oxidase did not mimic the hypoxic response, i.e. DPI did not enhance the expression of the above mentioned oxygen regulated genes under conditions of a high PO_2. We therefore hypothesize that hypoxia does not impair the electron flux through the oxidase. The observed decrease of H_2O_2 production in HepG2 cells [5] under hypoxia is most likely due to a decreased number of oxygen molecules available for reduction by the oxidase.

In addition, other oxygen sensing tissues seem to have a low output NAD(P)H oxidase as part of oxygen sensor. Mohazzab et al. [20] as well as Grimminger et al. [21] characterized – predominantly by means of DPI – an NAD(P)H oxidoreductase that was claimed to be involved in hypoxia sensing and specific signal transduction events underlying hypoxic pulmonary vasoconstriction. Fluorescence immunocytochemistry and light absorption photometry of rat lung slices revealed the presence of a unique cytochrome b_{245} containing NAD(P)H-oxidase protein in the walls of pulmonary arteries. It was concluded that this system may be involved in the initiation of hypoxic pulmonary vasoconstriction by a DPI-sensitive release of superoxide under hypoxic conditions [22].

Pulmonary neuroepithelial body cells (NEB) operating as chemoreceptors in airways and NEB-related tumors co-express mRNAs for both the hydrogen peroxide sensitive voltage gated potassium channel subunit KV3.3a and membrane components of NADPH oxidase [21]. Moreover, in chemoreceptor type I cells of the carotid body different components of the NADPH oxidase like $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ [22–25] are colocalized and may act as an oxygen sensor protein complex in control of ion channel activity and gene expression by O_2-dependent oxygen radical production [26,27]. However, wild-type B-cell lines as well as cytochrome $b_{558}(-)$ chronic granulomatous disease-derived B-cell lines, deficient in either $p22^{phox}$ or $gp91^{phox}$ of the high output NADPH oxidase, showed no impairment with respect to hypoxia- or $CoCl_2$-induced expression of VEGF and aldolase A [28]. This does not necessarily contradict a low output NAD(P)H oxidase as part of the actual oxygen sensor since it is not clear whether the low output oxidase is also affected by the mutations that lead to the disease with a loss of function of the high output oxidase in the B-cells used by Wenger et al. [28].

We found a significant decrease in the intracellular $^{\bullet}OH$ level of HepG2 cells after phenobarbital exposure. This might have been caused by a reduced ROS production rate by the different cytochromes or an altered Fenton reaction due to changes in Fe^{2+}/Fe^{3+} availability. Decreased $^{\bullet}OH$ level after phenobarbital treatment would then be responsible for the enhanced EPO synthesis without directly affecting an oxygen sensing cytochrome. Hydrogen peroxide (H_2O_2) detected in EPO-producing HepG2 cells [4] depressed the hypoxia stimulated EPO production [5]. The inhibition was antagonized by iron chelators and the $^{\bullet}OH$ scavengers dimethylthiourea or tetramethylthiourea [7] indicating the involvement of a Fenton-type reaction. Such a reaction was visualized in the perinuclear space of HepG2 cells [3,6]. Likewise, in primary rat hepatocytes H_2O_2 inhibited the insulin dependent expression of glucokinase mRNA induced by a low PO_2 and restored glucagon dependent phosphoenolpyruvate carboxykinase mRNA expression that was reduced under venous PO_2 in a perinuclear Fenton reaction [2,29].

On the molecular level the effects of H_2O_2 on EPO production have been attributed to an increase in the transcription factor hGATA2, which acts as an repressor in the EPO promoter in Hep3B cells [30]. In addition, H_2O_2 is described to affect the redox sensitive EPO mRNA binding protein [31] and to decrease the stability of hypoxia-inducible factor 1α protein (HIF-1α) [32]. HIF-1α and HIF-1β are essential components of the heterodimeric transcription factor HIF-1. HIF-1, the key regulator in the expression of PO_2 dependent genes, contains a $2Fe^{2+}$-cluster probably involved in the above described Fenton reaction [33].

Whereas the importance of ROS as second messengers for PO_2-dependent gene expression seems to find support from several groups working on the oxygen sensing field the identification of the primary oxygen sensing cytochrome producing in dependence of PO_2 ROS remains difficult.

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