Role of hydrogen peroxide in hypoxia-induced erythropoietin production

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The addition of exogenous H_2O_2 inhibited hypoxia-induced erythropoietin (Epo) production in the human hepatoma cell line HepG2. Likewise, elevation of endogenous H_2O_2 levels by the addition of menadione or the catalase inhibitor, aminotriazole, dose-dependently lowered Epo production. The inhibitory effect of exogenous H_2O_2 on Epo formation could be completely overcome by co-incubation with catalase. When GSH levels in HepG2 cells were lowered, Epo production was more susceptible to H_2O_2-induced inhibition, indicating that H_2O_2 might affect thiol groups in regulatory proteins. Endogenous production of H_2O_2 in HepG2 cells was dependent on the pericellular O_2 tension, being lowest under conditions of hypoxia. Our results support the hypothesis that an H_2O_2-generating haem protein might be part of the O_2 sensor that controls Epo production. High H_2O_2 levels under conditions of normoxia suppress, whereas lower levels in hypoxic cells allow *epo* gene expression.

INTRODUCTION

Erythropoietin (Epo) is the predominant regulator of erythropoiesis. Its production is greatly enhanced in kidney and liver cells when the Epo-producing cells become hypoxic as a result of anaemia or lowered inspiratory gas O_2 tension (Jelkmann, 1992). Hypoxia induces transcription of the *epo* gene. *In vitro* Epo mRNA levels are increased 50–100-fold in hypoxic human hepatoma cells (Goldberg et al., 1991; Fandrey and Bunn, 1993). Regulation of the *epo* gene is by a 29 bp enhancer element, located 3′ of the *epo* gene, which is responsible for hypoxia-induced transcription of the gene (Beck et al., 1993; Wang and Semenza, 1993). This enhancer acts in concert with promoter sequences 5′ to the *epo* gene (Blanchard et al., 1993). Evidence that the regulatory enhancer sequences may be part of a ubiquitous O_2 sensor was provided by Maxwell et al. (1993). An intracellular signal generated by a cellular O_2 sensor in response to changes in ambient O_2 tension could, via proteins bound to the enhancer element, alter the transcriptional rate of O_2 tension regulated genes (Maxwell et al., 1993).

A haem protein has been proposed as the O_2 binding part of the O_2 sensor (Goldberg et al., 1988). In analogy to haemoglobin function, it was hypothesized that this haem protein might change its conformational state depending on the ambient O_2 tension and transmit this conformational change via an as yet unknown intracellular signal (Goldberg et al., 1988). Based on studies with the Epo-producing human hepatoma cell line HepG2, a well-established cellular model for hypoxia-induced Epo production, participation of a *b*-type cytochrome in the O_2-sensing process has been proposed (Fandrey et al., 1990; Görlach et al., 1993). Spectral-photometric analysis of HepG2 cells revealed a *b*-type cytochrome that was insensitive to cyanide and changed its conformational state in response to changes in ambient O_2 tension (Görlach et al., 1993). Antibodies against the 22 kDa small subunit and cytosolic activating factor (p47) of NADPH oxidase recognized proteins of similar size in HepG2 cells (Görlach et al., 1993). The presence of an NADPH oxidase-like haem protein was further supported by the production of H_2O_2 in HepG2 cells (Görlach et al., 1993). As H_2O_2 is freely diffusible within the cell, it is a possible candidate for an intracellular messenger molecule.

The aim of the present study was to investigate the influence of H_2O_2 on Epo production in HepG2 cells. Levels of Epo mRNA and secreted protein were measured after the addition of exogenous H_2O_2 or increased production of endogenous H_2O_2. Furthermore, H_2O_2 production under different O_2 tensions was investigated. On the basis of our results we suggest that high H_2O_2 levels at high O_2 tension suppress Epo production whereas low levels under conditions of hypoxia allows a high rate of synthesis of Epo.

MATERIALS AND METHODS

Cell cultures and incubations

HepG2 cells from the American Type Culture Collection (ATCC no. HB8065) were maintained in RPMI 1640 medium (Flow Laboratories, Meckenheim, Germany) supplemented with 10% fetal bovine serum (Gibco, Eggenstein, Germany) and $NaHCO_3$ (2.2 g/l) in a humidified atmosphere (5% CO_2 in air) at 37 °C (Heraeus Incubators, Hanau, Germany). Cell monolayers were grown to confluence in 24-well polystyrene dishes (Falcon, Becton Dickinson, Heidelberg, Germany) and had a density of 5×10^5 cells/cm^2 at the beginning of the experiments. The medium (0.5 ml/cm^2) was renewed 24 h before the experiment. H_2O_2 or catalase in the respective experiments were directly added to the culture medium at the beginning of the 24 h experiment. Cells pretreated with buthionine sulphoximine (BSO) to reduce endogenous GSH levels (Kinnula et al., 1992) received the compound 24 h before and during the experiments.

Endogenous H_2O_2 production in HepG2 cells was increased by the direct addition of menadione for the 24 h experimental period (Kinnula et al., 1992). Menadione efficacy was confirmed by measurement of endogenous H_2O_2 production in HepG2 cells (see below). In most of the cultures not treated with menadione, the amount of H_2O_2 released into the supernatant was below the limit of detectability in our assay. Menadione-treated cells released measurable amounts of H_2O_2 even under hypoxic conditions (see also Figure 4).

Degradation of H_2O_2 by HepG2 cells was studied in the absence or presence of aminotriazole added to the culture medium for the 24 h experiments. Aminotriazole is an irreversible inhibitor of catalase in the presence of H_2O_2 (Margoliash et al., 1960). The efficacy of the aminotriazole treatment was confirmed

Abbreviations used: Epo, erythropoietin; BSO, buthionine sulphoximine.

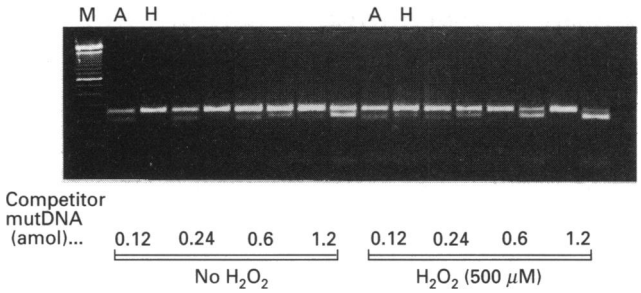

Figure 1 H_2O_2 inhibition of the hypoxia-induced increase in Epo mRNA levels in HepG2 cells

Normoxic HepG2 cells were made hypoxic for 1 h in the absence or presence of H_2O_2 (500 μM). Results from a representative quantification of Epo mRNA by competitive PCR are shown on a 2.5% agarose gel stained with ethidium bromide (0.5 μg/ml). Lane M represents the molecular mass marker (100 bp ladder). Lanes are arranged in pairs of products from one PCR reaction that were digested with *Acc*I (lane A) or *Hin*dIII (lane H). The amount of competitor mutDNA added to the PCR reactions ranged from 0.12 to 1.2 amol. The equivalence point for control cells is at 0.6 amol, and that for cells treated with H_2O_2 between 0.12 and 0.24 amol of competitor mutDNA.

by measuring the degradation of exogenously added H_2O_2 to HepG2 cells in the absence or presence of aminotriazole.

For 1 h experiments, cells were first exposed to 95% O_2 and 5% CO_2 for 6 h to overcome the hypoxia due to diffusion-limited O_2 supply (Wolff et al., 1993). During this preincubation, Epo mRNA levels decline to baseline levels (Fandrey and Bunn, 1993). At the beginning of the experiment, fresh prewarmed medium with or without H_2O_2 (500 μM) was added and the cells were switched to 1% O_2, 94% N_2 and 5% CO_2.

Biochemical determinations

For determination of Epo protein the medium was collected and frozen at −20 °C. The cell layer was washed with PBS and lysed with SDS/NaOH (5 g/l SDS in 0.1 M NaOH). Total cellular protein was determined by the method of Lowry et al. (1951) using a micro determination kit (Sigma Diagnostics, Taufkirchen, Germany).

GSH content was determined in homogenates of the cultures by the kinetic method of Brehe and Burch (1976).

Cytotoxicity was assessed by means of the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl]tetrazolium bromide assay performed exactly as recently described (Wolff and Jelkmann, 1993).

Quantification of Epo mRNA by competitive PCR

At the end of the 1 h experiments, cells were washed with sterile PBS and lysed with 4 M guanidinium isothiocyanate/0.1 M 2-mercaptoethanol. Total RNA was isolated by CsCl centrifugation as described (Chirgwin et al., 1979), redissolved in water and the concentration determined by measuring A_{260}.

Competitive PCR was performed as recently reported (Fandrey and Bunn, 1993). In brief, 1 μg of total RNA was reverse transcribed into first-strand cDNA using oligo$(dT)_{15}$ as a primer for reverse transcriptase (M-MLRV RT Superscript; Gibco). The efficiency of reverse transcription was determined as described (Fandrey and Bunn, 1993). PCR was performed in PCR buffer [50 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatine] containing 200 μM each dNTP, 300 nM each 5′ and 3′ primer and 5 units/ml *Taq* polymerase (Perkin–Elmer, Ueberlingen, Germany) in a final volume of 100 μl. The 5′ primer (5′-CTGCTCCACTCCGAACAATCAC-3′) contained nucleotides 2651–2672 of the GenBank file HUMERPA (accession no. M11319; Lin et al., 1985), and the 3′ primer (5′-CTGGAGTGTCCATGGGACAG-3′) contained nucleotides 2885–2904. cDNA (1 μl) of unknown concentration and 1 μl of a dilution series containing known amounts of the mutant competitor (mutDNA) were added to each tube. The mutDNA competitor was prepared from full-length genomic DNA in which the *Acc*I restriction site in exon V was deleted and a *Hin*dIII site created a few base pairs upstream. PCR products amplified from wild-type cDNA can only be cut with *Acc*I whereas mutDNA-derived products can only be cut by *Hin*dIII. PCR was run for 35 cycles after an initial denaturation at 95 °C with an amplification profile for each cycle consisting of denaturation for 1 min, primer annealing for 1.5 min and elongation for 3 min.

Equal volumes of PCR products were cut with either *Acc*I or *Hin*dIII, run on a 2.5% agarose gel and made visible by ethidium bromide (0.5 μg/ml) staining. A representative gel is shown in Figure 1.

H_2O_2 release from HepG2 cells

The dependence of H_2O_2 formation on pericellular O_2 tension was studied in confluent HepG2 cultures grown on Petri dishes with a gas-permeable hydrophilic fluorethylene–propylene copolymer Teflon membrane (thickness 25 μm; cell growth area 20.6 cm^2; cell density 5×10^5 cells/cm^2; Petriperm, Bachofer, Reutlingen, Germany). In these dishes, the pericellular O_2 partial pressure is very similar to that in the incubation gas (Wolff et al., 1993). Cells were preincubated with medium containing aminotriazole (50 mM) for 60 min to inhibit endogenous catalase. The medium was then replaced by Krebs–Ringer buffer (2 ml per dish; 140 mM NaCl, 4.9 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 6 mM NaH_2PO_4, 6 mM glucose; pH 7.2) containing 0.6 mM menadione and 50 mM aminotriazole. The cultures were incubated for 120 min in a closed chamber through which water-saturated gases with the desired O_2 concentrations were pumped (Wolff et al., 1993). Immediately thereafter, portions of supernatant (50 μl) were transferred to microtitre plates and 200 μl of assay buffer [100 mg/l 2,2′-azino-di(3-ethylbenzthiazoline-sulphonate) (Boehringer, Mannheim, Germany), 100 mM NaCl, 50 mM NaH_2PO_4, pH 4.4; and 2.5 units/ml horseradish peroxidase (Boehringer, grade 1)] was added. After 30 min incubation at room temperature the A_{405} was read (Easy Reader EAR 400 FW; SLT, Groedig, Austria). The H_2O_2 concentration of the samples was calculated from H_2O_2 standard curves (stock 3% H_2O_2 solution; Sigma) in the range 0–1000 μM. The lower detection limit of the assay was 4 μM H_2O_2.

Degradation of exogenous H_2O_2 in HepG2 cultures

Confluent HepG2 cultures grown in 24-well polystyrene dishes were incubated at 37 °C with 1 ml of culture medium to which 1 mM H_2O_2 (Perhydrol 30%; Merck, Darmstadt, Germany) was added. At distinct time intervals up to 90 min, supernatant was taken for assay of H_2O_2 as described above. H_2O_2 degradation was studied in the absence or presence of 50 mM aminotriazole.

RESULTS

HepG2 cells were grown in 24-well polystyrene dishes as a monolayer to a density of 5×10^5 cells/cm^2. At this density HepG2 cells incubated in an atmosphere of 5% CO_2 in air are hypoxic because of the diffusion-limited O_2 supply (Wolff et al., 1993) and therefore produce 10–15 units of Epo/h per g cell of protein.

Table 1 Inhibition of hypoxia-induced EPO production by H_2O_2 and enhancement of the inhibition by pretreatment with BSO

Data are the means ± S.D. of four to six separate cultures during a 24 h experiment. *Statistically significant difference compared with control cultures (H_2O_2: 0 μM) with $P < 0.05$ (Dunnett's test). Cultures pretreated with BSO (0.5 mM) received the compound 24 h before and during the experimental period.

	Epo production (% of control cultures)				
H_2O_2 (μM)...	0	10	30	100	300
No BSO pretreatment	100 ± 12	100 ± 12	91 ± 10	62 ± 2*	37 ± 5*
BSO pretreated	104 ± 14	85 ± 13	75 ± 8*	44 ± 19*	24 ± 13*

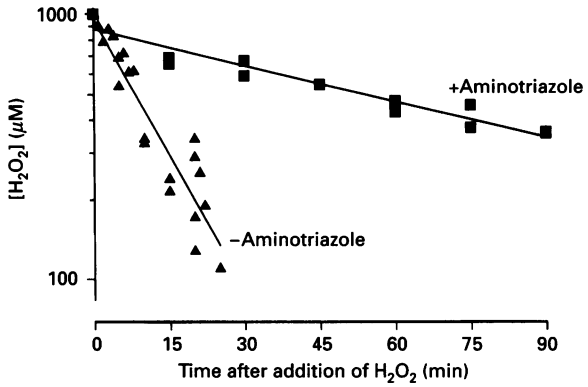

Figure 3 HepG2 cells treated with aminotriazole (50 mM) for 2 h to inhibit endogenous catalase displayed a markedly delayed degradation of exogenously added H_2O_2 compared with untreated controls

H_2O_2 was measured as described in the Materials and methods section.

H_2O_2 added to HepG2 cells for 24 h dose-dependently inhibited hypoxia-induced Epo production (Table 1). Cytotoxicity tests performed in parallel cultures demonstrated that there was no toxicity up to 500 μM H_2O_2. At 1 mM H_2O_2, a roughly 12% decrease in the cells' capacity to reduce formazan was observed, thus indicating some impairment of cellular integrity.

Enhancement of the H_2O_2-induced inhibition was achieved when endogenous GSH levels were lowered by preincubation with 0.5 mM BSO for 24 h (Table 1). The efficacy of BSO pretreatment was confirmed by measurement of the levels of GSH at the end of the experiment (control: 12.0 ± 1.8 μmol/g of cell protein; +0.5 mM BSO: 1.0 ± 0.5 μmol/g of cell protein; $P < 0.05$; means ± S.D.; $n = 6$).

Exogenous catalase dose-dependently antagonized the inhibitory action of H_2O_2 (1 mM) during a 24 h experiment. Complete prevention of the H_2O_2-dependent inhibition of Epo production was achieved with 100 μg/ml catalase [control: 329 ± 7 units of Epo/g of cell protein; + H_2O_2 (1 mM): 50 ± 5 U Epo/g of cell protein; + H_2O_2 (1 mM) and catalase (100 μg/ml: 318 ± 25 U Epo/g of cell protein; means ± S.D.]

The decrease in Epo protein production was accompanied by lower Epo mRNA levels in H_2O_2-treated cells than in control cultures [control cells: 0.6 amol/μg of total RNA; + H_2O_2 (500 μM) for 1 h: 0.24 amol/μg of total RNA; two experiments; see also Figure 1]. In these 1 h experiments, normoxic cells were rapidly switched to hypoxia to turn on *epo* gene transcription in the absence or presence of H_2O_2. It is noteworthy that H_2O_2 did not only inhibit accumulation of Epo protein during a 24 h experimental period but also prevented increase in Epo mRNA levels in the cells caused by exposure to hypoxia.

Endogenous H_2O_2 production in HepG2 cells was stimulated by the addition of menadione which undergoes intracellular redox cycling and increases the H_2O_2 production (Kinnula et al., 1992). Menadione dose-dependently inhibited hypoxia-induced Epo production during a 24 h experiment (Figure 2a). Likewise, when the intracellular degradation of H_2O_2 by catalase was blocked by the addition of aminotriazole, Epo production was inhibited (Figure 2b). Both compounds displayed no cytotoxicity in the dose range used. The efficacy of aminotriazole (50 mM) in inhibiting endogenous catalase activity was confirmed by the delayed degradation of exogenous H_2O_2 compared with untreated controls (Figure 3).

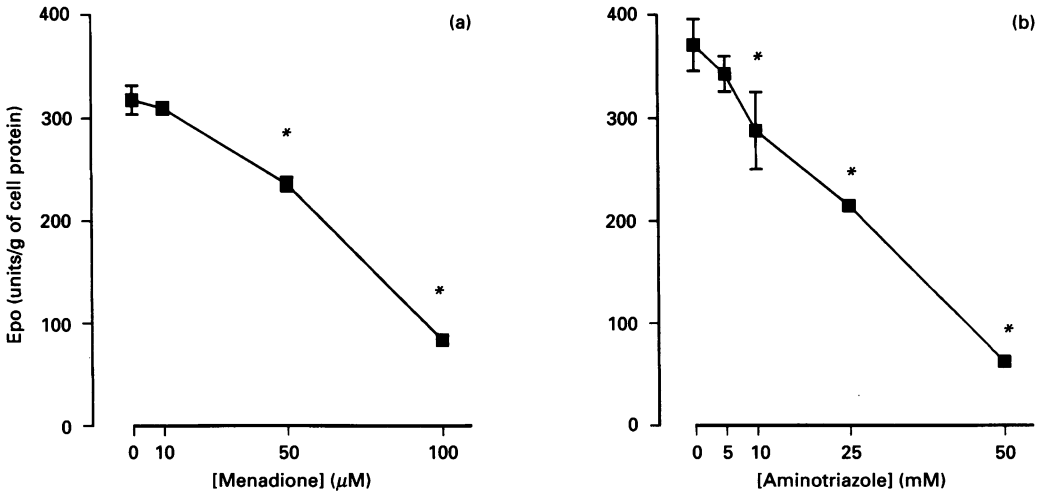

Figure 2 Inhibition of hypoxically induced Epo production by HepG2 cells by menadione (a) or aminotriazole (b) during a 24 h experiment

Data are means ± S.D. of four to six separate cultures. *Statistically significant difference from untreated controls ($P < 0.05$; Dunnett's test).

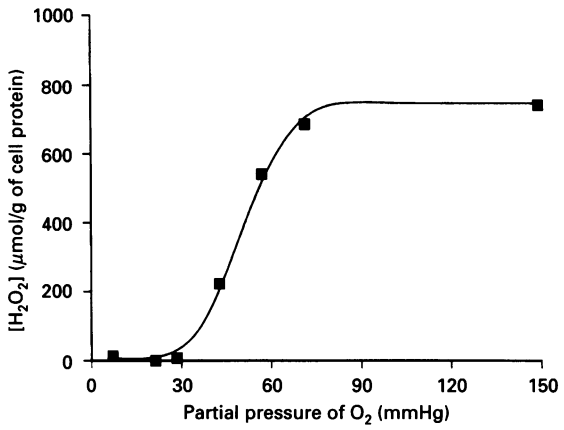

Figure 4 O_2-dependence of H_2O_2 production in HepG2 cells

HepG2 cells were grown on culture dishes with a gas-permeable membrane to ensure a pericellular O_2 partial pressure equal to that in the incubation chamber. H_2O_2 production was stimulated with menadione (600 μM) for 2 h at different O_2 partial pressures and measured as described in the Materials and methods section.

Endogenous H_2O_2 production by HepG2 cells was studied in menadione-stimulated cultures grown on gas-permeable supports to ensure equal pericellular and ambient O_2 tensions. As shown in Figure 4, a sigmoid curve representing the H_2O_2-production rate versus pericellular O_2 tension was obtained.

DISCUSSION

Absorbance maxima typical of a *b*-type cytochrome, the presence of several components of the NADPH oxidase complex and detection of H_2O_2 by rhodamine fluorescence strongly suggest that HepG2 cells have a cyanide-insensitive electron-transfer chain similar to the NADPH oxidase in neutrophils (Görlach et al., 1993).

Our study presents evidence that HepG2 cells produce H_2O_2 depending on the O_2 tension. We propose that H_2O_2 controls Epo production and thus participates in the O_2-sensing process. As first suggested by Sies (1977), several recent studies have demonstated the important role of H_2O_2 in other O_2-sensor systems. Spectral analyses and the fluorescence microscopic demonstration of H_2O_2 production have identified an H_2O_2-generating haem protein as a possible O_2 sensor in carotid-body preparations (Cross et al., 1990). Youngson et al. (1993) have reported that pulmonary neuroepithelial bodies produce H_2O_2 and are histochemically stained with an antibody recognizing the p91 polypeptide of the haem-linked NADPH oxidase. In addition, blockade of H_2O_2 production in cells containing neuroepithelial bodies mimics the reduction in the K^+ current that is normally seen under conditions of hypoxia in these cells (Youngson et al., 1993). Omar et al. (1993) have shown that H_2O_2 induces relaxation of pulmonary arteries via catalase-dependent activation of a guanylate cyclase. Under conditions of hypoxia, when H_2O_2 production is low, decreased cyclic GMP levels lead to pulmonary vasoconstriction (Omar et al., 1993).

Our results suggest that a similar O_2-sensing system may control Epo production in HepG2 cells. The hypothesis that an NADPH-like oxidase is part of the O_2 sensor is in line with results from earlier studies on the regulation of Epo production: the participation of a haem protein (Goldberg et al., 1988); the modulation of hypoxia-induced Epo production by inducers and inhibitors of a *b*-type cytochrome, the *P*-450 system (Fandrey et al., 1990); the sensitivity towards O_2 but not cyanide which excludes respiratory-chain type *b* cytochromes (Görlach et al., 1993). In addition, we provide evidence that H_2O_2 is a possible candidate for a freely diffusible signalling molecule between the sensor and the transcriptional activator(s). Elevated cellular H_2O_2 levels inhibited Epo protein production and the hypoxia-induced increase in Epo mRNA levels, mimicking high pericellular O_2 tension. Endogenous H_2O_2 production decreased when the pericellular O_2 tension was lowered. It is noteworthy that the steep slope of the sigmoid H_2O_2-production/O_2 partial pressure curve coincided with the O_2 partial pressure range at which Epo mRNA levels and Epo-production rate rise exponentially (Fandrey and Bunn, 1993; Wolff et al., 1993). For the same range of O_2 partial pressure values, Cross et al. (1990) observed the maximal increase in carotid-body chemoreceptor discharge.

The mechanism by which H_2O_2 inhibits *epo* gene expression remains to be elucidated. Recently, it has been shown that H_2O_2-induced activation of NF-κB, the oxidative stress-responsive transcription factor (Meyer et al., 1993), is counterbalanced by transcription factors that are activated by antioxidants that induce a cellular state similar to that of hypoxia (Meyer at al., 1993). Although the flanking regions of the *epo* gene contain NF-κB consensus sequences, the functional significance of these DNA elements has still to be determined.

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