Involvement of a local Fenton reaction in the reciprocal modulation by O_2 of the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase gene and the insulin-dependent activation of the glucokinase gene in rat hepatocytes

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H_2O_2 mimicked the action of periportal pO_2 in the modulation by O_2 of the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase (PCK) gene and the insulin-dependent activation of the glucokinase (GK) gene. H_2O_2 can be converted in the presence of Fe^{2+} in a Fenton reaction into hydroxyl anions and hydroxyl radicals ($\cdot OH$). The hydroxyl radicals are highly reactive and might interfere locally with transcription factors. It was the aim of the present study to investigate the role of and to localize such a Fenton reaction. Hepatocytes cultured for 24 h were treated under conditions mimicking periportal or perivenous pO_2 with glucagon or insulin plus the iron chelator desferrioxamine (DSF) or the hydroxyl radical scavenger dimethylthiourea (DMTU) to inhibit the Fenton reaction. PCK mRNA was induced by glucagon max-

imally under conditions of periportal pO_2 and half-maximally under venous pO_2 . GK mRNA was induced by insulin with reciprocal modulation by O_2 . DSF and DMTU reduced the induction of PCK mRNA to about half-maximal and increased the induction of GK mRNA to maximal under both O_2 tensions. Hydroxyl radical formation was maximal under arterial pO_2 . Perivenous pO_2 , DSF and DMTU each decreased the formation of $\cdot OH$ to about 70 % of control. The Fenton reaction could be localized in a perinuclear space by confocal laser microscopy and three-dimensional reconstruction techniques. In the same compartment, iron could be detected by electron-probe X-ray microanalysis. Thus a local Fenton reaction is involved in the O_2 signalling, which modulated the glucagon- and insulin-dependent PCK gene and GK gene activation.

INTRODUCTION

In the liver, the periportal and perivenous hepatocytes receive different signal patterns, since substrates (including oxygen and hormones) are degraded and products and mediators are formed during the passage of blood through the liver [1]. The periportal-to-perivenous drop in oxygen tension was considered to be a key regulatory factor in the zoned expression of carbohydrate-metabolizing enzymes. Thus the resulting different metabolic capacities of periportal and perivenous cell types led to the model of 'Metabolic Zonation' [2]. The gene for the glucogenic rate-controlling enzyme phosphoenolpyruvate carboxykinase (PCK) was found to be expressed predominantly in the more aerobic periportal area; conversely, the glycolytic enzyme glucokinase (GK) was found mainly in the less aerobic perivenous area [1,2]. In agreement with this model, in primary rat hepatocyte cultures the glucagon-dependent activation of the PCK gene was maximal under conditions of arterial pO_2 and only half-maximal under conditions of venous pO_2 [3–5]; insulin activated the gene of perivenous GK with reciprocal modulation by O_2 [6]. In hepatoma cell lines, genes encoding glycolytic enzymes such as aldolase A, lactate dehydrogenase and phosphoglycerate kinase 1, as well as the erythropoietin (EPO) gene, are activated by hypoxia [7–9].

Haem-containing oxidases producing H_2O_2 as a second mess-

enger have been proposed as oxygen sensors in hepatoma cells [10,11] and hepatocytes [5]. It was shown that, in hepatoma cells, H_2O_2 mimicked conditions of arterial pO_2 by destabilizing the α -subunit of the transcription factor HIF1 (hypoxia-inducible factor 1) [12], which led to an inhibition of the hypoxia-dependent increase in EPO mRNA [10]. In primary rat hepatocytes H_2O_2 mimicked conditions of periportal pO_2 in the modulation by O_2 of the glucagon-dependent activation of the PCK gene and of the insulin-dependent activation of the GK gene [5,6]. H_2O_2 , which is a non-charged molecule, can cross membranes and participate in one-electron transfer reactions. It can be subsequently converted non-enzymically in the presence of Fe^{2+} in a Fenton reaction into hydroxyl anions and highly reactive hydroxyl radicals ($\cdot OH$). It was proposed from studies investigating hypoxia-dependent EPO gene expression in HepG2 cells that such a Fenton reaction might be involved in the O_2 signalling pathway [13]. Thus the cells could react immediately via the Fenton reaction to modulate their pattern of gene expression in response to the prevailing pO_2 by interference by the highly reactive hydroxyl radicals with transcription factors such as HIF1 and activator protein 1 (AP-1), which have been shown to be regulators of O_2 -dependent gene expression.

If a Fenton reaction is involved in the O_2 signalling cascade, conditions mimicking venous pO_2 should reduce the intracellular level of hydroxyl radicals. It might be possible to inhibit the

Abbreviations used: AP-1, activator protein 1; DHR, dihydrorhodamine 123; DMTU, dimethylthiourea; DSF, desferrioxamine; EPO, erythropoietin; GK, glucokinase; HIF1, hypoxia-inducible factor 1; PCK, phosphoenolpyruvate carboxykinase; PGK, phosphoglycerate kinase; SNARF, seminaphthorhodafluor.

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production of hydroxyl radicals by short-term (2 h) treatment with iron chelators and hydroxyl radical scavengers, which should result in conditions mimicking venous pO_2 . It was the aim of the present study to measure the hydroxyl radical content in primary rat hepatocytes under conditions mimicking arterial or venous pO_2 , as well as in the presence of the iron chelator desferrioxamine (DSF) and the hydroxyl radical scavenger dimethylthiourea (DMTU). Additionally, DSF and DMTU were used to test the involvement of a Fenton reaction in the reciprocal modulation by O_2 of the glucagon-dependent activation of the PCK gene and the insulin-dependent activation of the GK gene in primary rat hepatocyte cultures. Furthermore, the intracellular localization of the Fenton reaction and the presence of iron were investigated by confocal laser microscopy [14] and electron probe X-ray microanalysis [15] respectively. It was found that perivenous pO_2 , DSF and DMTU reduced the formation of hydroxyl radicals, and that DSF and DMTU mimicked venous pO_2 in the reciprocal modulation by O_2 of the glucagon-dependent activation of the PCK gene and the insulin-dependent activation of the GK gene. Confocal laser microscopy and three-dimensional reconstruction showed that such a Fenton reaction takes place in a perinuclear space. Furthermore, iron could be detected in the same compartment by electron probe X-ray microanalysis. Thus it seems likely that a local Fenton reaction is involved in the O_2 -sensing signal chain that modulates the glucagon- and insulin-dependent activation of the PCK and GK genes.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and were purchased from commercial suppliers. Collagenase, digoxigenin-11-UTP, the digoxigenin nucleic acid detection kit, fetal calf serum and T3 RNA polymerase were from Boehringer (Mannheim, Germany). Medium M199 was from Gibco BRL (Eggenstein, Germany). Hormones were from Serva (Heidelberg, Germany). Guanidinium isothiocyanate was purchased from Fluka (Neu-Ulm, Germany), and nitrocellulose BA-S 85 was from Schleicher und Schüll (Dassel, Germany). Hyperfilm was from Amersham Buchler (Braunschweig, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Animals

Male Wistar rats (200–260 g) were kept on a 12 h day/night cycle (light from 07:00 to 19:00 h), with free access to water and food. Rats were anaesthetized with pentobarbital (60 mg/kg body weight), prior to preparation of hepatocytes between 08:00 and 09:00 h.

Cell culture experiments

Liver cells were isolated by collagenase perfusion. Cells (1×10^6 per dish) were maintained under standard conditions in an atmosphere of 16% O_2 , 79% N_2 and 5% CO_2 in medium M199 containing 0.5 nM insulin (added as a growth factor for culture maintenance), 100 nM dexamethasone (required as a permissive hormone) and 4% (v/v) fetal calf serum for the initial 4 h of culture. Cells were then cultured in serum-free medium from 4 to 24 h at 16% O_2 (mimicking arterial pO_2). At 24 h, the medium was changed and induction of PCK and GK was started by adding 0.1 nM glucagon or 1 nM insulin respectively either with or without 130 μ M DSF, 500 μ M DMTU and/or 50 μ M H_2O_2 ,

each at 16% O_2 or at 8% O_2 (the latter mimics venous pO_2). The O_2 values take into account the O_2 diffusion gradient from the medium surface to the cells [4].

RNA preparation and Northern analysis

Total RNA was prepared from 3×10^6 cells as described [5]. Samples of 10–20 μ g of RNA were denatured with formaldehyde and used in a typical Northern blot experiment. Digoxigenin-labelled antisense RNAs served as hybridization probes; these were generated by *in vitro* transcription from pBS-PCK or pBS-GK using T3 RNA polymerase and an RNA labelling mixture containing 3.5 mM digoxigenin-11-UTP, 6.5 mM UTP, 10 mM GTP, 10 mM CTP and 10 mM ATP. Hybridizations were carried out with 50 ng/ml transcript at 68 °C for 6 h according to the manufacturer's application notes of the digoxigenin nucleic acid detection kit (Boehringer Mannheim). Detection of hybrids was performed as described previously [6]. Blots were quantified using a videodensitometer (Biotech Fischer, Reiskirchen, Germany).

Measurement of $\cdot OH$ formation mediated by the Fenton reaction

$\cdot OH$ production was measured by monitoring the conversion of non-fluorescent dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR, U.S.A.) into fluorescent rhodamine 123. DHR is a non-fluorescent agent and has been described as a probe for the detection of H_2O_2 . It is well established that the reaction with H_2O_2 is rather slow under ambient conditions. It has been shown that the addition of iron compounds yields a fast oxidation of DHR to rhodamine 123 [16]. This hints at the involvement of the Fenton reaction in the activation process of DHR. A Fenton reaction oxidation involves several steps with well characterized kinetics. One important intermediate is the hydroxyl radical ($\cdot OH$), which has been detected by several spectroscopic methods [17]. Ehleben et al. [14] proposed the following chemical reaction for the conversion of non-fluorescent DHR into rhodamine 123. The reaction of $\cdot OH$ with DHR with the production of water yields a tertiary free radical which is known to be rather stable. The radical is able to produce a rearrangement of the π -system, yielding fluorescent rhodamine 123. The electron in excess can be abstracted either by another $\cdot OH$, yielding a hydroxyl anion (OH^-), or by H_2O_2 with the additional production of $\cdot OH$. The latter reaction pathway would be the starting point for a new cascade.

A total of 2×10^4 hepatocytes per well were cultured under standard conditions in microtitre plates. After 24 h of culture, the medium was removed and cells were washed three times with 0.9% NaCl. Then the culture was continued in Krebs/Ringer buffer containing 50 μ M DHR under conditions mimicking arterial or venous pO_2 . After 2 h the cells were washed and rhodamine 123 fluorescence was measured in a Fluoroscanner microplate reader (Flow Laboratories, Meckenheim, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

For three-dimensional visualization of the $\cdot OH$ distribution inside hepatocytes, optical sections were prepared as described by Ehleben et al. [14] and examined using a commercial confocal laser scanning microscope (MRC 600; Bio-Rad, Hempel Hempsted, U.K.) attached to an inverted microscope (ICM 405; Zeiss, Cologne, Germany) with a 60 $\times$ objective (CF N Plan Apo-chromat; Nikon GmbH, Düsseldorf, Germany) with cover slip correction, a numerical aperture of 1.20 and a working distance of 220 μ m. The optical sections were recorded with SOM software (Bio-Rad) and stored in a native PIC file format (Version 2.0). A

total of 39–42 optical sections with a resolution of 192×256 per channel were recorded, with a step size of $1 \mu\text{m}$. Typically, results from two pictures were averaged in order to minimize bleaching of the specimen. The volume elements producing $\cdot\text{OH}$ were stained with DHR ($50 \mu\text{M}$). Due to this specific staining, the limits of the cell could not be detected. In order to visualize $\cdot\text{OH}$ production as well as the extent of the cell, seminaphthorhodafluor (SNARF) calcein (Molecular Probes) at a concentration of $50 \mu\text{M}$ was used as a second dye; this showed minimal cross-talk with $\cdot\text{OH}$ labelling. The two dyes (DHR and SNARF calcein) were excited with the 488 nm line of an Ar ion laser (Omnichrome; Laser 2000, Wessling, Germany) and the fluorescence was detected using band pass filters of 570 nm and 640 nm respectively. Using this indicator, tandem cross-talk is minimized as far as the $\cdot\text{OH}$ imaging is concerned. Within the focal plane of the microscope, the power of the 488 nm line was reduced below 1 mW. The pinhole was adjusted to 1.9 mm; the photomultiplier gain was optimized for the individual channels; and an electronic zoom of 1.5–2.0 was used. The sequential 8-bit Bio-Rad PIC files were transferred to a UNIX-based workstation cluster via ethernet for image processing and data visualization. The processed data sets, as well as the real co-ordinate extents calculated using the physical characterization of the settings, were stored in the AVS field file format (Application Visualization System, Waltham, MA, U.S.A.) for processing on the SPARC 10 or INDY (Silicon Graphics Inc., Mountain View, CA, U.S.A.) equipped with 192 MB of RAM. We developed programs on the basis of AVS in order to perform the data visualization [18].

Electron probe X-ray microanalysis

Preparation and analysis of cryosectioned cells was carried out as previously described [15,19]. Cells grown on petriperm foil were cryofixed by plunging them into liquid propane cooled by liquid nitrogen. Cryosections of 100 nm were prepared in a Reichert FC4/Ultracut cryoultramicrotome (Reichert, Wien, Austria) using a dry glass knife at a temperature below 150 K. The cryosections were placed on pioloform-coated and carbon-evaporated Cu-grids and transferred into the cold stage of a Siemens ST 100F scanning transmission electron microscope (Siemens, Berlin, Germany). The sections were freeze-dried during cryotransfer. The freeze-dried cryosections were imaged and analysed by scanning an electron beam of 100 kV and 1.3 nA across the area of interest. X-rays were collected by means of a Si(Li) detector with a Be window (Nuclear Semiconductor, Menlo Park, CA, U.S.A.) and analysed with respect to their energy by a Link AN 10.000 multichannel analyser (Link, High Wycombe, Bucks., U.K.). From the X-ray, element contents related to dry mass were calculated by use of the Link Quantem FLS program based on the Hall continuum method. Element contents of cellular compartments depending on the particular experimental conditions were represented by mean values $\pm$ S.D.

RESULTS

Reduction of $\cdot\text{OH}$ formation by venous pO_2 , DSF and DMTU in primary rat hepatocyte cultures

In 24 h-cultured hepatocytes, $\cdot\text{OH}$ formation as an indicator of the Fenton reaction was measured over a time period of 6 h under conditions mimicking arterial and venous pO_2 . $\cdot\text{OH}$ formation remained constant at around near-maximal (100%) values under conditions mimicking arterial pO_2 , whereas under conditions of venous pO_2 the $\cdot\text{OH}$ content had decreased by

Figure 1 Decrease in $\cdot\text{OH}$ formation due to conditions of venous pO_2 in primary rat hepatocyte cultures: mimicry of venous pO_2 by DSF and DMTU

Hepatocytes were cultured for 24 h under conditions mimicking arterial pO_2 . (a) At 24 h the medium was changed and cells were further cultured for 6 h under conditions of either arterial (16% O_2) or venous (8% O_2) pO_2 . In each experiment the $\cdot\text{OH}$ level measured at 24 h under arterial pO_2 was set to 100%. Values are means $\pm$ S.E.M. of three independent culture experiments with 32 measurements per point. Student's *t*-test for paired values: * significant difference between values at 16% O_2 and 8% O_2 ($P < 0.01$). (b) At 24 h fresh medium containing 130 μM DSF or 500 μM DMTU was added for 2 h. In each experiment the $\cdot\text{OH}$ level measured in the control under arterial pO_2 was set to 100%. Values are means $\pm$ S.E.M. of three independent culture experiments with 32 measurements per point. Student's *t*-test for paired values: * significant difference compared with 16% O_2 ($P < 0.01$); ** significant difference between control and DSF- or DMTU-treated ($P < 0.01$).

about 60% within 6 h (Figure 1). Incubation of 24 h-cultured hepatocytes for 2 h with 130 μM DSF inhibited intracellular $\cdot\text{OH}$ formation by about 25% under arterial pO_2 ; under venous pO_2 the $\cdot\text{OH}$ content was not influenced by DSF (Figure 1). Treatment of 24 h-cultured hepatocytes with 500 μM DMTU, which acts as a $\cdot\text{OH}$ scavenger, reduced $\cdot\text{OH}$ formation to about 80% within 2 h. Again, DMTU did not influence $\cdot\text{OH}$ formation under venous pO_2 (Figure 1). Thus conditions mimicking venous pO_2 reduced $\cdot\text{OH}$ formation; in addition, impairment of the Fenton reaction by DSF or DMTU decreased $\cdot\text{OH}$ formation under arterial pO_2 , and mimicked the effects of venous pO_2 .

Inhibition by DSF of the glucagon-dependent induction of PCK mRNA and enhancement by DSF of the insulin-dependent induction of GK mRNA under conditions mimicking arterial pO_2

In 24 h rat hepatocyte cultures, glucagon induced PCK mRNA within 2 h maximally (to 100%) under conditions mimicking arterial pO_2 and to about 60% under venous pO_2 (Figure 2), which was in line with previous studies [3,4]. In the presence of 130 μM DSF, glucagon enhanced PCK mRNA to about 60% under both O_2 tensions (Figure 2). Reciprocally, insulin elevated GK mRNA within 3 h maximally to 100% under conditions mimicking venous pO_2 and to about 60% under arterial pO_2 (Figure 2), in agreement with a previous study [6]. Addition of 130 μM DSF elevated the insulin-dependent induction of GK mRNA to values of about 110% under both arterial and venous O_2 tensions (Figure 2). Basal PCK mRNA and GK mRNA levels were not influenced by treatment with DSF (Figure 2).

Thus glucagon enhanced PCK mRNA maximally under conditions mimicking arterial pO_2 and, reciprocally, insulin enhanced GK mRNA to higher values under venous pO_2 . Impairment of the Fenton reaction by the addition of DSF mimicked the effect of venous pO_2 ; in addition, the glucagon-dependent induction of PCK mRNA and the insulin-dependent induction of GK mRNA were no longer modulated by O_2 .

Figure 2 Mimicry of venous pO_2 by DSF in the reciprocal modulation by O_2 of the glucagon-dependent induction of PCK mRNA and the insulin-dependent induction of GK mRNA in primary rat hepatocytes

Hepatocytes were cultured for 24 h under conditions mimicking arterial pO_2 . PCK mRNA was induced by adding fresh medium containing 0.1 nM glucagon, and GK mRNA was induced without changing the culture medium by adding 1 nM insulin. Cells were then kept in the presence of 130 μ M DSF for 2 h (PCK) or 3 h (GK) under conditions of arterial (16% O_2) or venous (8% O_2) pO_2 . (a) In each experiment the maximum induction of PCK mRNA or GK mRNA measured by Northern blotting (cf. b) was set to 100%. Induction is the difference between induced and non-induced PCK mRNA and GK mRNA levels. Values are means $\pm$ S.E.M. of three independent culture experiments. Student's *t*-test for paired values: * significant difference between values at 16% O_2 and 8% O_2 ($P \leq 0.05$). (b) Representative Northern blot. In PCK experiments, 10 μ g of total RNA prepared from the cultured hepatocytes was hybridized to a digoxigenin-labelled PCK and β -actin antisense RNA probe (see the Materials and methods section), whereas in GK experiments 20 μ g of total RNA was analysed with a digoxigenin-labelled GK and β -actin antisense RNA probe. Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry. Ggn, glucagon; Ins, insulin.

Enhancement by H_2O_2 of the glucagon-dependent induction of PCK mRNA and inhibition by H_2O_2 of the insulin-dependent induction of GK mRNA under venous pO_2 : diminution of the action of H_2O_2 by DMTU

In 24 h rat hepatocyte cultures, glucagon induced PCK mRNA maximally to 100% under conditions mimicking arterial pO_2 and to about 60% under venous pO_2 , and insulin induced GK mRNA with reciprocal modulation by O_2 (Figures 2 and 3). In the presence of H_2O_2 , glucagon enhanced PCK mRNA to about 120% under arterial pO_2 and to about 110% under venous pO_2 (Figure 3). The hydroxyl radical scavenger DMTU reduced the glucagon-dependent induction of PCK mRNA to about 70% under arterial pO_2 and to about 50% under venous pO_2 (Figure

Figure 3 Simulation of arterial pO_2 by H_2O_2 in the reciprocal modulation by O_2 of the glucagon-dependent induction of PCK mRNA and the insulin-dependent induction of GK mRNA in primary rat hepatocytes: inhibition by DMTU

(a) Hepatocytes were cultured for 24 h under conditions mimicking arterial pO_2 . Then PCK mRNA was induced by adding fresh medium containing 0.1 nM glucagon, and GK mRNA was induced without changing the culture medium by adding 1 nM insulin. Cells were then kept in the presence of 50 μ M H_2O_2 and/or 500 μ M DMTU under arterial (16%) or venous (8%) pO_2 . Maximum PCK mRNA and GK mRNA levels were determined by Northern analysis 2 h and 3 h later respectively. In each control experiment without H_2O_2 , the maximal induced levels of PCK and GK mRNA were set to 100%. Induction is the difference between induced and non-induced PCK mRNA and GK mRNA levels. Values are means $\pm$ S.E.M. of three independent experiments. Student's *t*-test for paired values: * significant difference between values at 16% and 8% O_2 ($P \leq 0.05$). (b) Hybridization signals of total RNA prepared from glucagon-stimulated (top) and insulin-stimulated (bottom) cells, analysed as in Figure 2.

3). Addition of both H_2O_2 and DMTU abolished the H_2O_2 -mediated increase in the glucagon-dependent induction of PCK mRNA (Figure 3).

Addition of 50 μ M H_2O_2 reduced the insulin-dependent induction of GK mRNA to about 40% under venous pO_2 and to about 50% under arterial pO_2 (Figure 3). DMTU alone enhanced the insulin-dependent induction of GK mRNA to about 110% under both O_2 tensions. DMTU added together with H_2O_2 abolished the H_2O_2 -elicited decrease in the insulin-dependent induction of GK mRNA under venous pO_2 (Figure 3).

Thus the simulation of arterial pO_2 by H_2O_2 could be inhibited by the hydroxyl radical scavenger DMTU, which decreased the

Figure 4 Perinuclear localization of the local Fenton reaction degrading H_2O_2 in primary hepatocytes

Hepatocytes were cultured for 24 h under standard conditions, and were then incubated with the non-fluorescent dye DHR, which is converted into fluorescent rhodamine 123 by hydroxyl radicals generated in a Fenton reaction. (A, B) Three-dimensional reconstruction from two hepatocytes stained with DHR, indicating the local Fenton reaction. (C, D) Homologous distribution of rhodamine 123 fluorescence analysed as a control. Light blue indicates high fluorescence activity. The outer cell dimensions are indicated by isolines. The dimensions of the x, y and z axes are given in μm .

glucagon-dependent induction of PCK mRNA, increased the insulin-dependent induction of GK mRNA, and abolished the action of H_2O_2 and the modulation by O_2 .

Perinuclear localization of rhodamine fluorescence in primary rat hepatocytes

The non-fluorescent agent DHR reacts very poorly with H_2O_2 ; only in the presence of Fe^{2+} does a fast conversion of DHR into fluorescent rhodamine 123 occur [16], indicating the generation of $\cdot OH$ in a Fenton reaction [17]. The $\cdot OH$ reacts with DHR with the production of water and a tertiary radical which can rearrange the π -electron system, resulting in the generation of rhodamine 123 [14]. In the cell this rhodamine 123 fluorescence can be localized by confocal laser microscopy (Figures 4A and 4B). In 24 h-cultured hepatocytes, rhodamine 123 fluorescence was localized in a perinuclear space. The outer cell borders were visualized by staining with SNARF calcein, and are represented by isolines

after three-dimensional reconstruction (Figures 4A and 4B). Direct incubation with rhodamine 123 resulted in a homogeneous distribution inside the cytosol (Figures 4C and 4D). Thus in primary hepatocytes a Fenton reaction is localized in a perinuclear space, as indicated by the conversion of DHR into rhodamine 123.

Localization of iron-containing mass dense particles in the perinuclear zone of primary hepatocytes

Hepatocytes were cultured under standard conditions for 24 h. Then cells were cryofixed and the element content of subcellular compartments was determined by electron probe X-ray microanalysis. It was found that only mass dense particles located near the nucleus in the cytoplasm (Figure 5) contained the element Fe, which is necessary for the Fenton reaction (Table 1). These particles were further termed 'cytoplasmic mass dense particles'. The quantification of the Fe element in other subcellular com-

Figure 5 Localization of iron-containing mass dense particles in the perinuclear zone of a primary hepatocyte

Hepatocytes were cultured under standard conditions for 24 h. Then cells were cryofixed and scanned by electron probe X-ray microanalysis. (a) Scanning transmission electron micrograph of a freeze-dried cryosection. c, cytoplasm; g, cytoplasmic granule; m, mitochondrion; n, nucleus. (b) X-ray spectrum of the cytoplasmic granule (labelled g in the micrograph). The Fe peak represents 717 mmol of iron/kg dry mass. (c) X-ray spectrum of the mitochondrion (labelled m in the micrograph). (d) X-ray spectrum of the cytoplasm (labelled c in the micrograph).

Table 1 Element content in cellular compartments of primary rat hepatocytes

Rat hepatocytes were cultured on Petriperm foil. c_d is the element content in mmol/kg dry mass; results are means $\pm$ S.D. d , dry mass proportion; n , number of measurements, n.s., not significant. The element content in mmol/kg of water (c_w) can be obtained from the equation: $c_w = c_d \times d / (1 - d)$.

	c_d (mmol/kg dry mass)			
	Nucleus	Cytoplasm	Mitochondria	Cytoplasmic mass dense particle
Na	46 $\pm$ 10	31 $\pm$ 24	7 $\pm$ 3	55 $\pm$ 53
Mg	48 $\pm$ 12	46 $\pm$ 10	21 $\pm$ 5	20 $\pm$ 14
P	501 $\pm$ 45	288 $\pm$ 71	185 $\pm$ 40	195 $\pm$ 190
S	233 $\pm$ 27	163 $\pm$ 21	180 $\pm$ 9	222 $\pm$ 147
Cl	115 $\pm$ 18	98 $\pm$ 15	8 $\pm$ 4	41 $\pm$ 41
K	608 $\pm$ 43	374 $\pm$ 41	146 $\pm$ 21	156 $\pm$ 129
Ca	n.s.	n.s.	n.s.	n.s.
Fe	n.s.	n.s.	n.s.	228 $\pm$ 294
d	0.23	0.30	0.5	0.6
n	8	10	5	8

partments, such as the nucleus, mitochondria and cytoplasm, was not possible due to the detection limit of approx. 8 mmol/kg dry mass. Thus the perinuclear location of mass dense particles corresponded to the perinuclear localization of DHR conversion in the Fenton reaction.

DISCUSSION

The present investigation has shown that, in primary rat hepatocytes cultured under conditions mimicking arterial or venous pO_2 , the Fenton-reaction-mediated formation of $\cdot OH$ was maximal under arterial pO_2 and about half-maximal under venous pO_2 . Short-term treatment of hepatocytes cultured under arterial pO_2 with the iron chelator DSF or the hydroxyl radical scavenger DMTU resulted in the same decreases in $\cdot OH$ formation as were seen under venous pO_2 . The mimicry of venous pO_2 by DSF or DMTU was further observed in the reciprocal modulation by O_2 of the glucagon-dependent induction of PCK mRNA and the insulin-dependent induction of GK mRNA. In the presence of DSF and DMTU and under arterial pO_2 , glucagon induced PCK mRNA only to the lower venous values, whereas insulin elevated GK mRNA to the higher venous levels. The $\cdot OH$ -forming Fenton reaction could be localized by confocal laser microscopy to a perinuclear space. The element iron, which is necessary for the Fenton reaction, was localized in mass dense particles in a very similar compartment by electron probe X-ray microanalysis. Thus it seems likely that a local Fenton reaction is involved in the reciprocal modulation by O_2 of glucagon-activated PCK gene expression and insulin-activated GK gene expression.

Involvement of H_2O_2 -producing haem-containing oxidases in the response to O_2

O_2 -dependent H_2O_2 production has been reported in pulmonary neuroepithelial bodies [20], rat carotid body preparations [21], HepG2 cells [22], bovine pulmonary artery cells [23] and primary hepatocytes [5]. It appears that a haem-containing NADPH oxidase isoenzyme [24,25] of the cytochrome b type, similar to that in neutrophils [26], is involved in H_2O_2 production.

The hypothesis that an NADPH oxidase is part of the O_2 sensor is in line with results from this and earlier studies on the induction of the EPO, aldolase A and PCK genes. All investigations have shown the participation of a haem protein [4,5,9] and the simulation of arterial pO_2 by H_2O_2 [5,10,13]. This view was strengthened by the finding that the induction by hypoxia of EPO, vascular endothelial growth factor and glucose transporter 1 gene expression remained unaffected by Src kinase activity [27]. Thus a similar O_2 -sensing system involving redox-dependent reactions may control EPO production in HepG2 cells, as well as the modulation by O_2 of glucagon-induced PCK gene expression and insulin-activated GK gene expression in hepatocytes.

Mimicry of arterial pO_2 by H_2O_2

In neuroepithelial bodies, the hypoxia-dependent increase in K^+ current could be mimicked after the inhibition of H_2O_2 production [20]. Endogenously produced H_2O_2 allowed the relaxation of pulmonary arteries via the catalase-sensitive activation of a guanylate cyclase [23]. The hypoxia-induced increase in EPO mRNA in HepG2 cells [22] and the venous- pO_2 -induced elevation of aldolase A mRNA in primary hepatocytes were inhibited by 500 μM and 50 μM H_2O_2 respectively [5]. In Hep3B cells, H_2O_2 mimicked arterial pO_2 by destabilizing the α -subunit of the transcription factor HIF1 [12], thus inhibiting the hypoxia-dependent increase in EPO mRNA [10,13]. The glucagon-dependent induction of PCK mRNA under conditions of venous pO_2 was increased by 50 μM H_2O_2 to levels observed under arterial pO_2 in hepatocytes and, reciprocally, the same H_2O_2 concentration under venous pO_2 lowered the insulin-dependent induction of GK mRNA in hepatocytes to values obtained under arterial pO_2 (Figure 3). The H_2O_2 concentration of 50 μM did

not exert oxidative stress, since the oxidative stress responsive gene of haem oxygenase 1 was activated in similar hepatocyte cultures by concentrations of 100 μ M H₂O₂ and above, reaching a maximum at 200 μ M H₂O₂ [28].

H₂O₂ and its decomposition by the Fenton reaction

H₂O₂ is a non-charged molecule which can cross membranes and participate in one-electron transfer reactions, and it is therefore an ideal candidate for being a second messenger for O₂. Since H₂O₂ itself is a relatively inert reaction partner, its degradation products are of great importance. H₂O₂ is usually degraded in the cytosol and mitochondria by glutathione peroxidase and in peroxisomes by catalase [29], and can be non-enzymically converted in the presence of Fe²⁺ in a Fenton reaction into hydroxyl anions and hydroxyl radicals [30]. $\cdot$ OH is highly reactive and thus can act immediately at its site of production, as indicated by the local conversion of DHR into rhodamine (Figure 4). The effects of short-term treatment of hepatocytes with the iron chelator DSF on both the $\cdot$ OH content and the modulation by O₂ of PCK and GK gene expression were a good indicator that a Fenton reaction was involved. The formation of $\cdot$ OH was monitored under conditions of arterial and venous p O₂, and it was found that venous p O₂ reduced $\cdot$ OH formation to about 60% within 2 h. Under the same conditions the glucagon-dependent induction of PCK mRNA was maximal under arterial p O₂ (when the $\cdot$ OH formation was high) and only about half-maximal under venous p O₂ (when $\cdot$ OH formation was low). Inhibition of $\cdot$ OH formation by either DSF or DMTU under arterial p O₂ mimicked the effect of venous p O₂, reducing $\cdot$ OH formation and glucagon-dependent PCK gene activation. Thus the venous- p O₂-dependent reduction in $\cdot$ OH formation or the impairment of $\cdot$ OH formation by DSF or DMTU decreased glucagon-dependent PCK gene activation under conditions of arterial p O₂.

With the GK gene, it was found that insulin enhanced GK mRNA to maximal levels under conditions of venous p O₂ (when the $\cdot$ OH content was low). Inhibition of the Fenton reaction by DSF or of $\cdot$ OH formation by DMTU under arterial p O₂ again mimicked the effects of venous p O₂, leading to enhanced GK mRNA induction. Thus the venous- p O₂-dependent reduction in $\cdot$ OH formation, or the impairment of $\cdot$ OH formation by DSF or DMTU, increased insulin-dependent GK gene activation under conditions of arterial p O₂. In conclusion, PCK gene activation by glucagon and insulin-dependent GK gene activation were reciprocally modulated by the O₂-dependent $\cdot$ OH formation mediated by the Fenton reaction.

In agreement with these findings, iron-containing mass dense particles were found in an area around the nucleus in which the conversion of DHR into rhodamine could also be localized (Figures 4 and 5). Thus the cells could react adequately via the Fenton reaction to modulate their pattern of gene expression in response to the prevailing p O₂ by interference by the highly reactive hydroxyl radicals with Fe-S clusters, as with aconitase [31], or with cysteine residues in transcription factors such as HIF1 and AP-1 [22], which have been shown to be activators of low-O₂-dependent gene expression. This would suggest that, under conditions of high p O₂, where the $\cdot$ OH content is also high, both transcription factors have to be inactivated. These transcription factors might act as inhibitors of glucagon-dependent PCK gene activation under conditions of venous p O₂ (i.e. low $\cdot$ OH content), whereas they might enhance the insulin-dependent activation of the GK gene.

Mimicry of venous p O₂ by DSF

The strong activation of the EPO gene in Hep3B cells after a 24 h

exposure to 130 μ M DSF supported the proposal that a haem protein functions as the O₂ sensor [9]. This conclusion was based on the observation that, in primary embryonic chicken hepatocyte cultures, 178 μ M DSF inhibited the incorporation of radio-labelled 5-aminolaevulinate into haem by 51% within 3 h [32]. In contrast with the rather long-term effects of DSF in the studies with the EPO gene [33], in the present study with primary hepatocytes 130 μ M DSF decreased the $\cdot$ OH content and abolished the modulation by O₂ of the glucagon-dependent activation of the PCK gene and the insulin-dependent activation of the GK gene within 2 h and 3 h respectively (Figures 1 and 2). The mimicry of venous p O₂ by DSF may be explained mainly by the inhibition of $\cdot$ OH formation by Fenton-reaction-mediated mechanisms; inhibitory actions on haem synthesis could only be involved if the O₂-sensing haem protein has a high rate of turnover.

Mimicry of venous p O₂ by DMTU

The biochemically active molecule generated from H₂O₂ via the Fenton reaction appears to be $\cdot$ OH [30]. To support the notion that $\cdot$ OH is involved in the O₂ response, the influence of the $\cdot$ OH scavenger DMTU was investigated in the modulation by O₂ of glucagon-dependent PCK expression and insulin-dependent GK expression. DMTU is cell permeable and has been shown to effectively inhibit the action of $\cdot$ OH in mesangial cells [34] and in an *in vitro* assay [35]. The results of the present study are in line with a study with HepG2 cells, in which DMTU and tetramethylthiourea were shown to counteract the inhibition by H₂O₂ of hypoxia-dependent EPO production [13]. This further substantiates the view that H₂O₂ mediates its effect as a second messenger for O₂ by local generation of $\cdot$ OH radicals.

Involvement of transcription factors in the regulation of gene expression by the O₂/H₂O₂ system

The DNA binding of transcription factors can be influenced by H₂O₂ produced in response to the prevailing p O₂. Several lines of evidence suggest that the DNA binding of the oxidative stress responsive transcription factor nuclear factor κ B [36] is induced by H₂O₂, whereas binding of AP-1 [37], p53 [38], redox factor 1 [39] and HIF1 [40] was increased under reducing conditions. The role of H₂O₂ as a mediator of p O₂ and the involvement of subsequent redox-dependent reactions was supported by the finding that H₂O₂ could diminish HIF1 binding activity to the 3' EPO gene enhancer [40] and that H₂O₂ destabilized the α -subunit of the transcription factor HIF1 [12]. Thus the same mediator, H₂O₂, might contribute to the activation or repression of gene expression in response to the p O₂ via modulation of several transcription factors.

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