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Modulation of the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase gene by oxygen in rat hepatocyte cultures

Evidence for a heme protein as oxygen sensor

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The glucagon-dependent activation of the phosphoenolpyruvate carboxykinase (PCK) gene is modulated by oxygen. It was proposed that heme proteins might function as O_2 sensors; their actions are impaired after replacement of the central Fe^{2+} ion by Co^{2+} and inhibition of heme synthesis by succinylacetone (SA). Therefore, the effects of $CoCl_2$ and SA, alone and in combination, on the glucagon-dependent induction of PCK activity and PCK mRNA were investigated at different physiological oxygen tensions in primary rat hepatocyte cultures. The cells were exposed to 50 μM $CoCl_2$ and/or 2 mM SA from 4–24 h. After addition of fresh media without $CoCl_2$ or SA, PCK was induced with 1 nM glucagon. PCK activity and PCK mRNA were elevated to 100% at 16% O_2 and to about 65% at 8% O_2. $CoCl_2$ reduced these increases to about 45% at 16% O_2 and to about 35% at 8% O_2. SA lowered the inductions to about 50% and 40% each at 16% and 8% O_2. $CoCl_2$ plus SA diminished the elevations to about 5% at both oxygen tensions. In the presence of $CoCl_2$ and/or SA, ornithine decarboxylase induction by insulin was not impaired; lactate dehydrogenase did not leak from the cells, which in electron microscopical inspections had normal cell structures. These findings support the hypothesis that a heme protein is involved in the activation of the PCK gene and that it acts as an O_2 sensor.

Metabolic zonation; Phosphoenolpyruvate carboxykinase gene; Heme protein; Hepatic oxygen sensing; Glucagon.

1. INTRODUCTION

The cytosolic phosphoenolpyruvate carboxykinase (PCK) is a major rate-generating gluconogenic enzyme in parenchymal liver cells and epithelial cells of proximal kidney tubules. The occurence of the enzyme in the adipose tissue, mammary gland and small intestine has been described [1–6]. In liver parenchyma most key enzymes are heterogeneously distributed: the periportal or upstream and perivenous or downstream zone have different catalytic potentials. This is the basis of the model of metabolic zonation [7–9]. PCK is predominantly localized in the periportal zone similar to other enzymes involved in glucose formation, such as glucose 6-phosphatase, fructose 1,6-bisphosphatase, alanine aminotransferase and tyrosine aminotransferase. Conversely, enzymes participating in glucose utilization, such as glucokinase and pyruvate kinase, are preferentially situated in the perivenous area [7,8].

PCK activity does not appear to be regulated on the level at enzyme modification by phosphorylation/dephosphorylation but at the level of gene transcription influenced by several hormonal factors [10], cell–cell signalling [11,12], developmental requirements [13] and environmental factors [11]. Glucagon, via cAMP, activates PCK transcription under the permissive action of glucocorticoids and thyroid hormones while insulin is a transcription inhibitor [14–16].

During passage of blood through the liver concentration gradients of oxygen, substrates and hormones are established [7,8], which may be the cause for the differential expression of the genome in periportal and perivenous zones. It was shown in rat hepatocyte cultures that PCK induction by glucagon resulted in higher activity and protein levels under arterial than under venous oxygen tensions [17]. This induction was modulated on the level of gene transcription via an unknown oxygen sensing system [18]. It was proposed that a specific heme protein could act as O_2 sensor and modulate gene activation in liver [18], as may be the case with the erythropoietin gene in the kidney [19]. Therefore, the aim of this study was to test this hypothesis.

It was found in primary rat hepatocyte cultures that the modulation by oxygen of PCK gene activation was lost if the cells had been pretreated with $CoCl_2$ to replace the central Fe^{2+} in heme or with succinylacetone to inhibit heme synthesis. These findings support the idea that an O_2 sensing heme protein plays a role in the

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differential expression of PCK in the periportal and perivenous zone.

2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals were of analytical grade and purchased from commercial suppliers. Collagenase, cell culture medium M199, fetal calf serum and the digoxigenin nucleic acid detection kit were from Boehringer (Mannheim). Hormones were delivered from Serva (Heidelberg). Succinylacetone (SA) (4,6-dioxoheptanic acid), antifoam A and sarcosyl (*N*-lauroylsarcosine) were purchased from Sigma (Deisenhofen). Guanidinium thiocyanate was from Fluka (Neu-Ulm) and Nitrocellulose BA-S 85 from Schleicher & Schüll (Dassel). [^{14}C]L-Ornithine and [^{14}C]L-leucine were supplied by Amersham Buchler (Braunschweig). All other chemicals were from Sigma (Taufkirchen). The plasmid Bluescript-PCK (pBS-PCK) was constructed as described in [20].

2.2. *Cell culture and induction experiments*

Liver cells were isolated from male Wistar rats (200–260 g) by collagenase perfusion. Cells (about 1×10^6 per dish) were maintained under an atmosphere of 16% O_2, 79% N_2, and 5% CO_2 (by vol.) in medium M 199 containing 1 nM insulin (as growth factor for culture maintenance [21]) and 100 nM dexamethasone (as permissive factor [14]), and for the initial 4 h of culture 4% fetal calf serum. Fresh media containing 50 μM $CoCl_2$, 2 mM SA or both, were added after the initial 4 h up to 24 h. Induction of PCK was started at 24 h by adding fresh media with 1 nM glucagon either under 16% O_2, 79% N_2, and 5% CO_2 (by vol.), mimicking arterial tensions, or under 8% O_2, 87% N_2, 5% CO_2 (by vol.) mimicking perivenous oxygen tensions. These values take into account the O_2 diffusion gradient from the media surface to the cells [17]. For maximal induction of PCK mRNA cells were harvested after 2 h at 26 h and of PCK activity after 4 h at 28 h [18] (cf. Fig. 2). As a control ornithine decarboxylase (ODC) induction by 100 nM insulin was assayed under the same conditions [22].

2.3. *Cell viability*

The lack of an increase in lactate dehydrogenase activity in the culture medium served as an indicator of cell integrity. 60 μl of media were removed every 2 h. Measurements were performed in a standard optical test. As a second control parameter overall protein synthesis was estimated by incorporation of [^{14}C]leucine in TCA precipitable protein [23]. Cell morphology was controlled by scanning electron microscopy performed by a standard method.

2.4. *Enzyme assays*

PCK activity was determined in duplicate exactly according to [24]. ODC activity was measured with L-[1-^{14}C]ornithine as substrate according to [25] with modifications: cells of 3 dishes (3×10^6) were washed twice in ice-cold 0.9% NaCl and scraped in 700 μl lysis buffer (50 mM HEPES, 5 mM DTT, 0.5 mM pyridoxal phosphate, 0.1 mM EDTA, pH 7.1; stored at −20°C). The cell suspension was calibrated to equal volumes and freeze–thawed five times and then centrifuged at 17,000× g (4°C). 900 μl of the supernatant were transferred to the main compartment of a Warburg flask with one side-arm containing 500 μl 6 N HCl. The central well was filled with 200 μl 2 N KOH. The reaction was started by adding 100 μl L-ornithine (1 mM) containing (0.2 μCi) [^{14}C]L-ornithine and continued under shaking for 2 h at 37°C. The reaction was stopped by tipping in HCl from the side-arm. The mixture was then incubated for an additional 1 h. The KOH solution was used for radioactivity measurements in a Philips liquid scintillation counter.

2.5. *RNA preparation and Northern analysis*

Total RNA was prepared from 3×10^6 cells as described previously [21]. A digoxigenin (DIG)-labelled antisense PCK RNA from the pBS-PCK generated by in vitro transcription using T3 RNA polymerase and DIG-UTP (Boehringer, Mannheim) served as the hybridization probe. Hybridizations were carried out at 68°C according to manufacturer's application notes of the DIG-nucleic acid detection kit (Boehringer, Mannheim). Detection of hybrids was performed by enzyme-linked immunoassay using an anti-digoxigenin alkaline phosphatase conjugate (anti-DIG-AP). Hybrids were visualized either via color reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates or via chemiluminescence with 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) (Boehringer, Mannheim). Luminescent blots were exposed to Hyperfilm-MP (Amersham, Braunschweig) and quantified with a videodensitometer (Biotech-Fischer, Reiskirchen).

3. RESULTS

In primary rat hepatocyte cultures the PCK gene was activated by glucagon under an atmosphere of 16% and 8% O_2, mimicking arterial and venous tensions. The involvement of an O_2-sensing heme protein in gene activation was examined by pretreatment of the cultures with $CoCl_2$ and/or succinylacetone (SA) which impaired the action of heme proteins by replacing the central Fe^{2+} ion by Co^{2+} [19] and by inhibiting heme synthesis, respectively [26].

3.1. *Optimal Co^{2+} concentration and cell viability*

Increasing concentrations of $CoCl_2$ resulted in a hyperbolic repression of PCK induction by glucagon (Fig. 1). 50 μM $CoCl_2$ was chosen for further investigations. Cell viability was tested by lactate dehydrogenase (LDH) leakage, [^{14}C]leucine incorporation into TCA precitable protein and electron microscopical inspection of cellular structures. Up to concentrations of 300 μM $CoCl_2$ hepatocytes did not increase leakage of LDH within 24 h. In the presence of 50 μM $CoCl_2$ overall protein synthesis proceeded unaltered and the cellular integrity on the electron microscopical level was preserved. As an additional parameter the induction of ornithine decarboxylase (ODC) by insulin was investigated. In untreated cells insulin (100 nM) induced ODC 2.1-fold independent of the oxygen tensions. In $CoCl_2$-treated cells basal levels of ODC were doubled; insulin induced these elevated levels further by 2.1-fold irrespective of the O_2 tensions. These findings indicate that the normal cell viability was preserved during the treatment with $CoCl_2$.

3.2. *Optimal succinylacetone (SA) concentration and cell viability*

The optimal SA concentration was deduced from previous studies with murine erythroleukemia cells [26]. Exposure of these cells to 1 mM SA increased cell growth until day 3, but decreased heme concentrations to about one sixth. In the present experiments the SA concentration was doubled in order to see inhibitory effects in heme synthesis already after day 1. In SA treated hepatocyte cultures basal ODC levels were normal and induction of ODC by insulin was not affected. Thus SA had no adverse effects on cell viability.

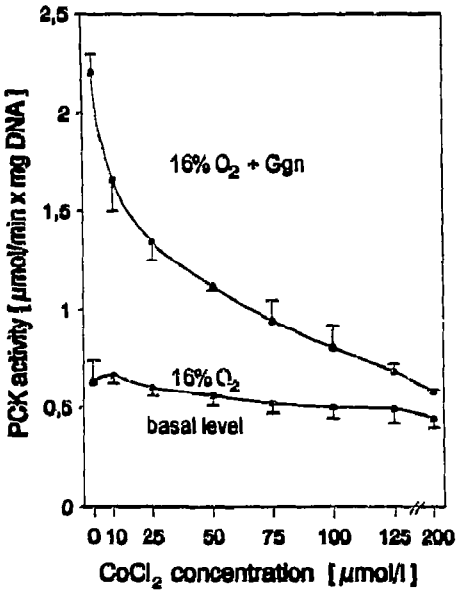

Fig. 1. Repression of the glucagon-dependent induction of PCK activity by $CoCl_2$ in rat hepatocyte cultures. Cells were exposed to the indicated concentrations of $CoCl_2$ for 24 h at 16% O_2. After a change to a $CoCl_2$-free medium 1 nM glucagon (Ggn) was added and PCK was measured at 28 h. Values are means of ± S.E.M. of 3 independent experiments.

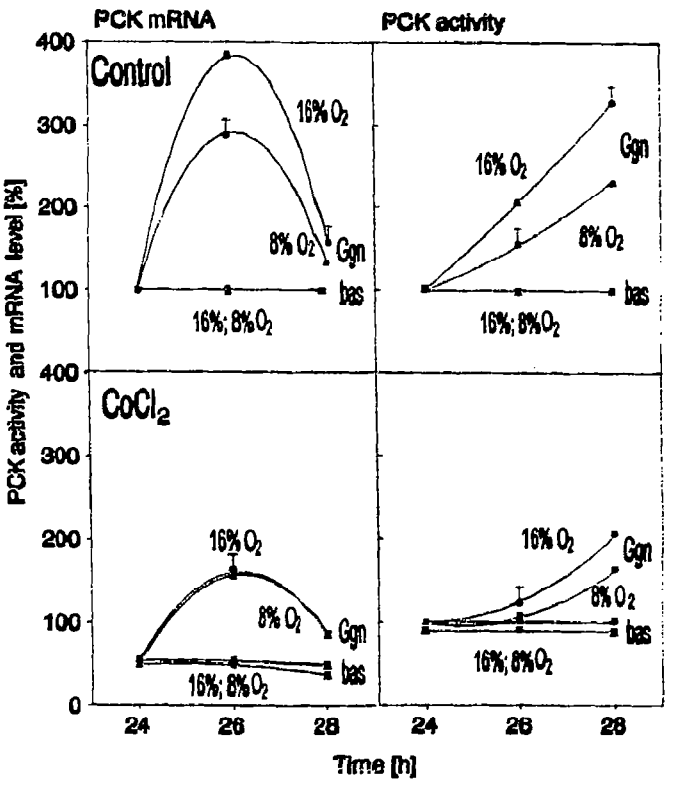

Fig. 2. Time-course of the glucagon-dependent increase in PCK mRNA and PCK activity in $CoCl_2$-pretreated hepatocyte cultures. Cells were exposed to 50 µM $CoCl_2$ when indicated for 24 h under 16% O_2. After a change to a $CoCl_2$-free medium 1 nM glucagon (Ggn) was added under 16% and 8% O_2. PCK mRNA and PCK activity were determined 2 h and 4 h later. In each control experiment without $CoCl_2$ the densitometric signal of basal (bas) mRNA and enzyme activity (in the range of 0.6 µmol/min x mg DNA) was set equal to 100%. Values are means ± S.E.M. of 3 independent culture experiments.

3.3. *Modulation by oxygen of the glucagon-dependent induction of PCK*

In rat hepatocyte cultures PCK mRNA was enhanced by glucagon to a maximum after 2 h; it then declined again. PCK enzyme activity was increased by the hormone to submaximal levels after 4 h (Fig. 2). These kinetics were in line with previous studies [18].

Glucagon induced PCK activity from 24 to 28 h of culture to the highest level = 100% at 16% O_2 and to a lower level = 60% at 8% O_2 (Fig. 3). After pretreatment of the cells from 4 to 24 h of culture with $CoCl_2$ these increases were reduced to 50% under 16% O_2 and to 30% under 8% O_2. The basal PCK activity was decreased only slightly (Fig. 2). Glucagon elevated PCK mRNA from 24 to 26 h of culture to the highest values = 100% under 16% O_2 and to smaller values = 67% under 8% O_2 (Fig. 3). After exposure of the cells to $CoCl_2$ from 4 to 24 h an induction of only 38% at both 16% and 8% O_2 could be measured. The basal PCK mRNA was decreased to one half (Fig. 2).

In cells pretreated from 4 to 24 h with 2 mM SA the PCK activity increase was only 50% both at 16% and 8% O_2 (Fig. 3); basal activity was reduced only slightly. PCK mRNA induction was reduced to 40% at both high and low oxygen tensions. The basal PCK mRNA was lowered to about one third.

The addition of both Co^{2+} and SA did not reduce basal enzyme activities but the PCK mRNA content was decreased to 50% under 16% and 8% O_2. Glucagon could not induce enzyme activity and mRNA under 16% O_2 and 8% O_2 (Fig. 3).

Thus, when the function of heme proteins was impaired either by replacing the central Fe^{2+} with Co^{2+} or by inhibiting the synthesis of heme, the glucagon-dependent activation of the PCK gene was reduced and no longer modulated by oxygen.

4. DISCUSSION

It was shown in the present investigation that in rat hepatocytes the activation of the PCK gene by glucagon was no longer modulated by oxygen when the cells were pretreated with Co^{2+} and/or succinylacetone, which replace the central Fe^{2+} of heme proteins or inhibit their synthesis, respectively. These findings indicate that a heme protein could function as the oxygen sensor. While many oxygen-modulated genes are known in prokaryotes and eukaryotes, the oxygen-sensing mechanism is not as yet well understood.

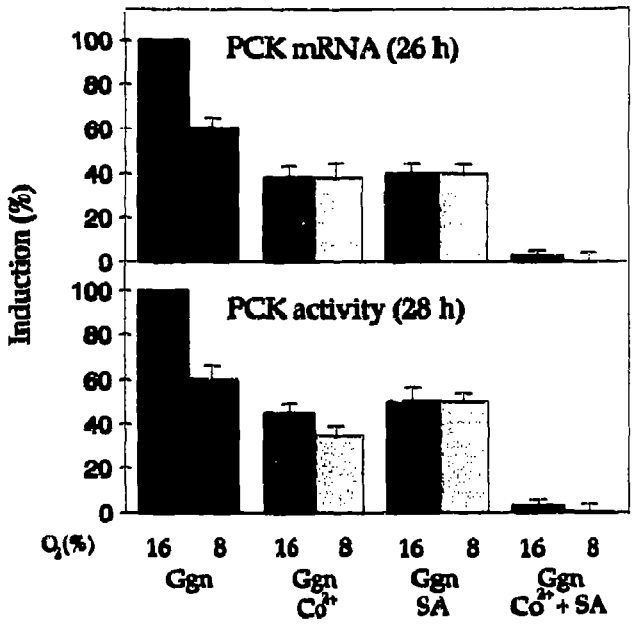

Fig. 3. Increase in PCK mRNA and PCK activity by glucagon in rat hepatocyte cultures pretreated with $CoCl_2$ and/or succinylacetone. Cells were exposed for 24 h under 16% O_2 to 50 μM $CoCl_2$ and/or 2 mM succinylacetone (SA) as indicated. After a change to a $CoCl_2$- or SA-free medium 1 nM glucagon (Ggn) was added under 16% and 8% O_2. PCK mRNA and PCK activity were determined 2 h and 4 h later, respectively (cf. Fig. 2). Induction is the difference between induced and non-induced mRNA levels or enzyme activities. The highest induction in the controls under 16%O_2 was set equal to 100%; it corresponded to a 3.8-fold increase in mRNA and a 3.2-fold increase in enzyme activity. Values are means ± S.E.M. of 4 independent culture experiments. SA = succinylacetone; Ggn = glucagon.

4.1. *Modulation of gene expression by oxygen*

The facultative anaerobic bacterium, *Escherichia coli*, possesses an aerobic respiratory chain containing a low O_2 affinity cytochrome *o* and high O_2 affinity cytochrome *d* and an anaerobic respiratory chain containing fumarate reductase or nitrate reductase. The gene for cytochrome *o* is repressed and the genes for cytochrome *d*, as well as for fumarate reductase and nitrate reductase, are induced by anoxia [27–29]. In the bacterium, *Rhizobium meliloti*, the nitrogen fixation genes are activated when the free oxygen concentration is reduced to microaerobic levels [30]. In the facultative aerobic yeasts, *Saccharomyces cerevisiae* and *Schwanniomyces occidentalis*, the aerobic respiratory cytochrome *c* 1 or 10, respectively, are down-regulated and cytochrome *c* 7 is up-regulated under hypoxic conditions [31,32].

In fetal liver and adult kidney the erythropoietin gene is activated, when the O_2 supply is decreased [19,33–36]. In endothelial cells expression of the platelet-derived growth factor β (PDGF-β) and angiotensin converting enzyme (ACE) gene is elevated by hypoxia [37]. In the carotid body tyrosine hydroxylase is enhanced with hypophysiological O_2 supply [37]. In HeLa cells the mitochondrially coded subunits of NADH dehydrogenase, cytochrome *c* oxidase and H^+-ATPase are repressed by hypoxia [38]. In liver the expression of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase [17,18,39] appears to be modulated positively and that of glucokinase and pyruvate kinase [39] negatively by oxygen. More examples of oxygen-regulated genes are known in mammals [37].

4.2. *Mechanisms of oxygen sensing*

In *E. coli* two transcriptional regulators controlling the activation/inactivation of O_2-dependent genes have been identified. One is the two-component arcB/arcA system, which under anaerobic conditions represses many aerobic enzymes and can induce anaerobic enzymes; the other is fnr, which represses some aerobic and induces anaerobic respiratory processes [27–29]. ArcB is a transmembrane and arcA a cytoplasmic protein [29]; fnr is a member of the growing family of cyclic AMP receptor protein (CRP)-related proteins, which have a DNA binding domain [27]. Less is known of the way in which arcB and fnr sense anoxia. With arcB it has been hypothesized that a so far unidentified reduced component of the respiratory chain is the activator and that the activated arcB phosphorylates arcA, the transcriptional regulator proper [29]. With fnr it seems likely that its reduced state with a cysteine-Fe^{2+} complex is the activated form [27].

In *Rhizobium meliloti* two regulator proteins, fixL and fixJ, initiate the expression of the nitrogen-fixation genes under microaerobic conditions. fixL is an O_2-binding heme protein, which senses O_2 through its heme moiety and transduces its signal by phosphorylating fixJ [30].

In *Saccharomyces cerevisiae* the gene coding for cytochrome *c* 1 (CYC 1) is transcribed under aerobic conditions or normal heme availability but not under anaerobic conditions or heme deficiencies. The gene is activated by a heme-activated protein (HAP1), which possesses a DNA binding cystein-rich zinc finger domain and a heme binding domain [40].

In fetal liver and adult kidney a heme protein in its deoxy form initiates the activation of the erythropoietin (EPO) gene in hypoxia [19]. The intermediate components of the signal chain from the O_2-sensing heme protein to the EPO gene are not known so far. Evidence for the involvement of the heme protein were the stimulation in Hep3B cells of the EPO gene expression by $CoCl_2$, which induces the deoxy conformation, and its inhibition by CO, which induces the oxy conformation [19].

In the present study with primary rat hepatocytes similiarly but conversly, the glucagon-dependent expression of the PCK gene was positively modulated by normoxia, when the sensor heme protein would be in the oxy form, and negatively modulated by hypoxia and by $CoCl_2$, when the heme protein would be in the deoxy form, as well as by succinylacetone, when the synthesis

of heme proteins would be impaired. Previous hypotheses that the oxygen sensor is the respiratory chain via cytochrome *c*, the ratio of oxidized to reduced glutathione or of disulfide to sulfhydryl groups, certain oxygenases with appropriate K_m values for oxygen and the lactate dehydrogenase isoenzymes [41] are not in line with the obtained data. Based on the present findings it is likely that hepatocytes sense oxygen via a heme protein. This heme protein my act directly as a transcription factor by binding to a defined DNA sequence, an oxygen regulatory element (ORE), of the PCK gene. The sensor heme protein may also act indirectly; it could be a protein kinase which phosphorylates either an oxygen transcription factor (OTF), which then binds to an ORE, or one of the known transcription factors, e.g. the cAMP regulatory element binding protein (CREB).

Thus it appears that oxygen, sensed via heme proteins, is the major signal for the zonation of gene expression in liver. Strong support for this proposal comes from the finding that in livers of mice, transgenic for the human erythropoietin gene, EPO mRNA was localized only in the perivenous zone when the animals were made anemic [35].

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