RESEARCH COMMUNICATION

Arterial oxygen partial pressures reduce the insulin-dependent induction of the perivenously located glucokinase in rat hepatocyte cultures: mimicry of arterial oxygen pressures by H_2O_2

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Liver glucokinase (GK) is localized predominantly in the perivenous zone. GK mRNA was induced by insulin maximally under venous O_2 partial pressure ($p\text{O}_2$) and only half-maximally under arterial $p\text{O}_2$. CoCl_2 and desferrioxamine mimicked venous $p\text{O}_2$ and enhanced the insulin-dependent induction of GK

mRNA under arterial $p\text{O}_2$. H_2O_2 mimicked arterial $p\text{O}_2$ and reduced insulin-induced GK mRNA under venous $p\text{O}_2$ to the lower arterial levels. Thus the zonal O_2 gradient in liver seems to have a key role in the heterogenous expression of the GK gene.

INTRODUCTION

The mammalian enzyme glucokinase (GK) is a key enzyme of glucose utilization; it is considered to play a crucial role in maintaining blood glucose homeostasis. In comparison with the product-inhibited hexokinases I–III with a K_m of about 20–50 μM , GK (hexokinase IV) has a K_m for glucose of about 10 mM and is not inhibited by its reaction product, glucose 6-phosphate [1,2]. These attributes allow the enzyme to react adequately to changes in the plasma glucose levels. GK is found only in hepatocytes, the pancreatic β -cells and some neuroendocrine cells of the central nervous system and gastrointestinal tract [3].

In liver, the periportal and perivenous parenchymal cells possess a different equipment with key enzymes, translocators, receptors and subcellular structures which led to the model of metabolic zonation [4]. GK and pyruvate kinase are predominantly localized in the perivenous cells which take up glucose from the blood. Reciprocally, the glucogenic enzymes phosphoenolpyruvate carboxykinase (PCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase are found to a higher extent in the periportal cells which release glucose [5]. During blood passage through the liver, concentration gradients of oxygen, substrates and products as well as hormones and mediators, are established. The O_2 partial pressure ($p\text{O}_2$) falls from about 65 mmHg (1 mmHg $\equiv$ 133.3 Pa) periportal to 35 mmHg perivenously and was considered to be an important factor in the zonal expression of the genes of carbohydrate-metabolizing enzymes [6]. It was shown in primary rat hepatocyte cultures that the gene for the periportal gluconeogenic enzyme PCK could be activated by glucagon to higher levels under arterial than under venous $p\text{O}_2$ [7]. In hepatoma cell lines, glycolytic-enzyme genes such as those coding for aldolase A (ALD A), lactate dehydrogenase (LDH A) and phosphoglycerate kinase 1, as well as the erythropoietin (EPO), were activated by hypoxia [8–10]. It was proposed that an H_2O_2 -generating haem protein is involved in the O_2 -sensing system, in both hepatocytes [11] and hepatoma cells [12].

It was the aim of the present study to investigate the influence of arterial and venous $p\text{O}_2$ and the potential role of H_2O_2 in the insulin-dependent induction of GK mRNA. It was found that insulin induced GK mRNA to higher levels under venous $p\text{O}_2$ and that CoCl_2 and desferrioxamine (DSF) mimicked venous $p\text{O}_2$ and H_2O_2 arterial $p\text{O}_2$. Thus the proposal that the O_2 gradient in liver plays a key role in the zoned expression pattern of the genes of carbohydrate-metabolizing enzymes and that an H_2O_2 -generating haem protein is involved in the O_2 -sensing system in response to O_2 was further substantiated.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and purchased from commercial suppliers. Collagenase, digoxigenin (DIG)-UTP, the DIG nucleic acid detection kit and fetal-calf serum were from Boehringer (Mannheim, Germany), medium M199 from Gibco BRL (Eggenstein, Germany) and T_3 RNA polymerase from United States Biochemicals. Hormones were from Serva (Heidelberg, Germany). Guanidinium thiocyanate was purchased from Fluka (Neu-Ulm, Germany) and nitrocellulose BA-S 85 from Schleicher und Schüll (Dassel, Germany). Hyperfilm and [^{14}C]leucine were supplied by Amersham Buchler (Braunschweig, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Animals

Male Wistar rats (200–260 g; Winkelmann, Borcheln, Germany) were kept on a 12 h day/12 h 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

Abbreviations used: $p\text{O}_2$, O_2 partial pressure (O_2 tension); GK, glucokinase; PCK, phosphoenolpyruvate carboxykinase; FBPase, fructose-1,6-bisphosphatase; ALD A, aldolase A; LDH A, lactate dehydrogenase; EPO, erythropoietin; DIG, digoxigenin; AC, β -actin; CoPP, Co^{2+} -protoporphyrin IX; HIF1, hypoxia-inducible factor 1; DSF, desferrioxamine.

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O₂/N₂/CO₂ (6:79:5) atmosphere in medium M 199 containing 0.5 nM insulin added as a growth factor for culture maintenance, 100 nM dexamethasone required as a permissive hormone and 4 % fetal-calf serum for the initial 4 h of culture. Cells were then cultured in serum-free medium from 4–24 h at 16 % O₂ (mimicking arterial pO₂). At 24 h, induction of GK was started by adding 1 nM insulin and, when indicated, 50 μ M CoCl₂, 130 μ M DSF, 50 μ M H₂O₂ and/or 30 μ g/ml catalase, each at 16 % O₂ or at 8 % O₂ (mimicking venous pO₂). The O₂ values take into account the O₂ diffusion gradient from the media surface to the cells [13].

RNA preparation and Northern analysis

Total RNA was prepared from 3×10^6 cells as described in [7]. A 15 μ g portion of RNA was denatured with formaldehyde and used in a typical Northern experiment. DIG-labelled antisense GK and β -actin (AC) RNA served as hybridization probes; they were generated by *in vitro* transcription from pBS-GK1, pBS-AC using T₃ RNA polymerase and DIG-UTP. Hybridizations were carried out with 50 ng/ml transcript at 68 °C for 6 h according to the manufacturer's application notes for the DIG-nucleic acid detection kit (Boehringer-Mannheim). Detection of hybrids was performed as described previously [11]. Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen, Germany).

Miscellaneous

The following parameters of cell integrity were considered: LDH activity in the culture medium was measured in a standard optical test. Overall protein synthesis was estimated by incorporation of [¹⁴C]leucine into trichloroacetic acid-precipitable protein [11]. Cell morphology was monitored by optical microscopy.

RESULTS

In primary rat hepatocytes, the GK gene was activated by insulin under arterial and venous pO₂ in order to examine the modulation of insulin-dependent mRNA induction by O₂. A ferrohaem-containing H₂O₂-producing oxidase had been proposed to be involved in the O₂-sensing system. To test for such an involvement, the cells were treated with CoCl₂, DSF, H₂O₂ and/or catalase.

Positive modulation by venous pO₂ of the insulin-dependent induction of GK mRNA

In 24 h primary rat hepatocytes the expression of GK mRNA was induced by increasing insulin concentrations from 0.1 nM to 100 nM under both O₂ partial pressures; GK mRNA was nearly undetectable in the absence of insulin. Over a range of physiological insulin concentrations from 0.1 nM to 1 nM the insulin-dependent induction was about 2-fold higher under venous pO₂ than under arterial pO₂. At insulin concentrations of 10 nM, GK mRNA was induced to about the same levels under both venous and arterial pO₂. Insulin concentrations of 100 nM increased GK mRNA to higher levels under arterial pO₂ than under venous pO₂ (Figure 1). Thus, under physiological conditions, the insulin-dependent induction of GK mRNA was positively modulated by venous pO₂, and an insulin concentration of 1 nM was chosen for further experiments.

Figure 1 Insulin-dependent induction of GK mRNA in primary rat hepatocytes cultured under arterial and venous pO₂

Hepatocytes were cultured for 24 h under arterial pO₂. GK mRNA was induced without changing the culture medium by adding the insulin (Ins) concentrations indicated. Cells were then kept for 3 h under arterial and venous pO₂. (a) In each experiment the highest induction of GK mRNA by 100 nM insulin measured by Northern blotting (cf. b) was set equal to 100%. Values are means $\pm$ S.E.M. for three independent culture experiments. Student's *t* test for paired values: *significant differences 16% O₂ versus 8% O₂, $P \leq 0.05$. (b) Representative Northern blot. A 20 μ g portion of total RNA prepared from the cultured hepatocytes was hybridized to DIG-labelled GK and AC antisense RNA probes (cf. the Materials and methods section). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry.

Enhancement by CoCl₂ and DSF of the insulin-dependent induction of GK mRNA under arterial pO₂

In 24 h rat hepatocyte cultures, insulin induced GK mRNA to a maximum within 3 h; thereafter GK mRNA declined again (Figure 2). Insulin elevated GK mRNA within 3 h maximally to 100% under venous pO₂ and to a lower level of about 60% under arterial pO₂ (Figure 2). Basal values of GK mRNA were hardly detectable during the entire 6 h period investigated. In the presence of 50 μ M CoCl₂, insulin enhanced GK mRNA to about 80% under both venous and arterial pO₂ (Figure 2). Addition of 130 μ M DSF elevated the insulin-dependent induction of GK mRNA to values of about 110% each under arterial and venous pO₂ (Figure 2). The basal GK mRNA levels were not induced by treatment with either CoCl₂ or DSF (Figure 2).

Thus insulin enhanced GK mRNA to higher values under venous pO₂ compared with arterial pO₂. Impairment of haem proteins caused by the addition of CoCl₂ and DSF mimicked venous pO₂, and the insulin-dependent induction of GK mRNA was no longer modulated by O₂.

Inhibition by H₂O₂ of the insulin-dependent induction of GK mRNA under venous pO₂

In 24 h rat hepatocyte cultures insulin induced GK mRNA maximally to 100% under venous pO₂ and to 60% under arterial pO₂. Addition of 50 μ M H₂O₂ reduced the insulin-dependent induction of GK mRNA to about 40% under venous pO₂ and to

Figure 2 Time course of the insulin-dependent induction of GK mRNA in CoCl_2 - and DSF-treated hepatocytes under arterial and venous $p\text{O}_2$

Cells were cultured for 24 h under arterial $p\text{O}_2$. At 24 h, 50 μM CoCl_2 or 130 μM DSF were added either with or without 1 nM insulin (Ins) and the cells were further cultured under arterial and venous $p\text{O}_2$. (a) In each control experiment the maximal induced level of GK mRNA measured by Northern blotting (cf. b) was set equal to 100%. Induction is the difference between induced and non-induced GK mRNA levels. Values are means $\pm$ S.E.M. for four independent culture experiments. Statistics: Student's t test for paired values: *significant differences 16% O_2 versus 8% O_2 , $P \leq 0.05$. (b) Representative Northern blot. A 20 μg portion of total RNA was analysed as described in the legend to Figure 1. Only the hybridization signals from the maximal induction at 3 h are shown.

about 50% under arterial $p\text{O}_2$ (Figures 3a and 3b). The basal GK mRNA levels were not influenced by treatment with H_2O_2 (Figure 3b).

The specificity of the H_2O_2 effects were tested by adding catalase to the culture media in the presence and absence of H_2O_2 . Catalase alone did not alter the modulation by O_2 of the insulin-dependent induction of GK mRNA. Catalase added together with H_2O_2 abolished the H_2O_2 -elicited decrease in the insulin-dependent induction of GK mRNA under venous $p\text{O}_2$ (Figure 3a and 3b). Therefore H_2O_2 acted directly, and not indirectly, by increasing the extracellular and then the intracellular $p\text{O}_2$.

Thus H_2O_2 simulated arterial $p\text{O}_2$. It decreased the insulin-dependent induction of GK mRNA under venous $p\text{O}_2$ to the lower arterial values and abolished the modulation by O_2 .

DISCUSSION

Regulation of metabolism and metabolic enzyme gene expression by O_2

Gluconeogenesis is an endergonic process; it is driven by the oxidation of fatty acids and therefore should occur in zones of high $p\text{O}_2$, i.e. periportal. Glycolysis is an exergonic process; it is not dependent on O_2 and therefore could take place in zones of low $p\text{O}_2$, i.e. perivenously [5,6]. As expected from these thermodynamic considerations, gluconeogenesis takes place in the periportal high- $p\text{O}_2$ zone and glycolysis in the perivenous low $p\text{O}_2$ area [14]. This reciprocal zonal localization is in accord with the short-term regulation of the two antagonistic processes by

Figure 3 Modulation by O_2 of the insulin-dependent increase of GK mRNA

Simulation of arterial $p\text{O}_2$ by H_2O_2 . Primary hepatocytes were cultured for 24 h under arterial $p\text{O}_2$. At 24 h, cells were treated with 50 μM H_2O_2 and/or 30 mg/ml catalase and GK mRNA was induced by the addition of 1 nM insulin. Cells were then further cultured under arterial and venous $p\text{O}_2$. GK mRNA was determined at its maximum, 3 h later, by Northern analysis (cf. b). (a) In each control experiment without H_2O_2 the maximal induced level of GK mRNA was set equal to 100%. Induction is the difference between induced and non-induced GK mRNA levels. Values are means $\pm$ S.E.M. for three independent experiments. Student's t test for paired values: *significant difference between 16% and 8% O_2 , $P \leq 0.05$. (b) Northern blot. A 20 μg portion of total RNA was analysed as described in the legend to Figure 1.

O_2 . In periportal-like hepatocytes, net gluconeogenic flow increased and in perivenous-like cells net glycolytic flow decreased, with increasing $p\text{O}_2$ [15].

The present study has shown that the periportal-to-perivenous O_2 gradient in liver is not only a key regulator for the long-term expression of a key enzyme gene of gluconeogenesis, PCK, but also for that of glycolysis, GK. In cultured hepatocytes, glucagon enhanced transcription within 0.5 h, mRNA abundance within 2 h and PCK activity within 4–6 h to maximal levels under arterial, and to half-maximal levels under venous, $p\text{O}_2$ [7]. The mirror image was demonstrated in the present study by the finding that insulin, in physiological concentrations, enhanced the induction of GK mRNA within 3 h maximally under venous $p\text{O}_2$ and half-maximally under arterial $p\text{O}_2$ (Figure 1).

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

Mimicry of venous $p\text{O}_2$ by CoCl_2 and DSF

The strong activation of the EPO gene in Hep3B cells after 24 h exposure to CoCl_2 led to the proposal that a haem protein functions as the O_2 sensor [10]. This was found also for other genes such as the vascular-endothelial-growth-factor gene in Hep3B cells [16] and the PCK gene in primary hepatocytes, in which the modulation by O_2 of the glucagon-dependent activation was lost after treatment for 24 h with CoCl_2 [17]. Co^{2+} ions have been shown to be a substrate for ferrochelatase and therefore to be incorporated into the porphyrin moiety, resulting in Co^{2+} -protoporphyrin IX (CoPP) which cannot bind O_2 [18]. In livers of rats injected with 250 μmol of CoCl_2/kg body wt. the total haem content was reduced after 1 h to about

72 % and the CoPP content was increased to about 28 %; the activity of the key enzyme of haem synthesis, 5-aminolaevulinic synthase, was decreased after 1 h to about 45 % [18]. Similar effects of 30 μ M CoCl₂ were observed also in primary embryonic chicken hepatocyte cultures [19]. In the same cultures, 178 μ M DSF inhibited the incorporation of radiolabelled 5-aminolaevulinic into haem by 51 % within 3 h [20]. Thus the mimicry of venous pO_2 by CoCl₂ and DSF in the present study (Figure 2) may be explained by their inhibitory actions on haem synthesis provided that the haem protein involved in the O₂-sensing system has a rapid turnover.

Mimicry of arterial pO_2 by H₂O₂

The blockade of H₂O₂ production in neuroepithelial bodies mimicked the reduction in the K⁺ current, which is normally seen under hypoxic conditions [21]. In pulmonary arteries, endogenously produced H₂O₂ induced relaxation via a catalase-sensitive activation of a guanylate cyclase [22]. The hypoxia-induced increase in EPO mRNA in HepG2 cells [12], and the venous pO_2 -induced elevation of ALD A mRNA in primary hepatocytes were inhibited by 500 μ M and 50 μ M H₂O₂ respectively [11]. The glucagon-dependent induction of PCK mRNA under venous pO_2 was increased by 50 μ M H₂O₂ to the levels observed under arterial pO_2 in hepatocytes [11]. Reciprocally, in this study the same H₂O₂ concentration under venous pO_2 lowered the insulin-dependent induction GK mRNA in hepatocytes to values obtained under arterial pO_2 (Figure 3). The H₂O₂ concentration of 50 μ M did not exert oxidative stress, since the oxidative-stress-responsive gene of haem oxygenase 1 was activated in the same hepatocyte cultures starting with 100 μ M H₂O₂, reaching a maximum at 200 μ M H₂O₂ [23].

H₂O₂-generating haem proteins as O₂ sensors

H₂O₂ production dependent on pO_2 has been reported for pulmonary neuroepithelial bodies [21], rat carotid-body preparations [24], HepG2 cells [12], primary hepatocytes [11] and bovine pulmonary artery cells [22]. In the first three preparations a haem-containing NADPH oxidase of the cytochrome *b*-type similar to that in neutrophils, which is composed of gp91, p47_{phox}, p67_{phox} and p22_{phox} [25], appears to be involved. The finding that, in B-cell lines deficient in gp91 and p22_{phox} derived from a patient suffering from chronic granulomatosis the O₂-sensing mechanism was unimpaired [26] supported the role of an NADPH oxidase isoenzyme in O₂-sensing. The hypothesis that an NADPH oxidase is part of the O₂ sensor is in line with results from the present and previous studies on the induction of the GK as well as the EPO, ALD A and PCK genes. All investigations have shown the participation of a haem protein [10,17,27] and the stimulation of arterial pO_2 by H₂O₂ [11,12]. Thus a similar O₂-sensing system may control EPO production in HepG2 cells as well as the modulation by O₂ of the glucagon-induced PCK gene, insulin-induced GK gene expression and the low O₂-dependent expression of the ALD A gene in hepatocytes.

Regulation of gene expression by H₂O₂ via oxidation–reduction of transcription factors

H₂O₂ produced in response to the pO_2 could influence the DNA binding of transcription factors. DNA binding of NF κ B, the oxidative stress-responsive transcription factor [28], is induced by

H₂O₂, whereas binding of activator protein 1 [29], p53 [30], redox factor 1 [31], hypoxia-inducible factor 1 (HIF1) [32] was increased under reductive conditions. The finding that H₂O₂ could diminish HIF1 binding activity to the 3'-EPO gene enhancer [32] would be in line with the proposal that H₂O₂ could be a mediator of the pO_2 . Thus several transcription factors modulated by the same mediator H₂O₂ might contribute to the activation or repression of gene expression in response to the pO_2 .

This study was supported by the Deutsche Forschungsgemeinschaft SFB 402. We thank Professor D. K. Granner (Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, U.S.A.) for kindly providing GK1 cDNA.

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