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Regulation of the gluconeogenic phosphoenolpyruvate carboxykinase and the glycolytic aldolase A gene expression by O_2 in rat hepatocyte cultures. Involvement of hydrogen peroxide as mediator in the response to O_2

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Abstract Heme proteins acting as oxidases which produce H_2O_2 have been proposed to function as O_2 sensors. In order to find out whether the modulation by O_2 of PCK gene activation and the stimulation of the ALD A gene by venous O_2 operate via H_2O_2, the effects of different concentrations of H_2O_2 and catalase as H_2O_2 scavenger were studied in rat hepatocyte cultures under different O_2 tensions. Primary hepatocytes were treated with 0.1 nM glucagon, 50 μM H_2O_2 and/or 100 μg/ml catalase each at arterial O_2 or venous pO_2. PCK mRNA was induced by glucagon maximally under arterial O_2 and only half maximally under venous O_2. ALD A mRNA was induced only by venous O_2. H_2O_2 enhanced the induction of PCK mRNA to similar levels under both O_2 tensions and the induction of ALD A mRNA under venous O_2 was completely inhibited. Addition of catalase antagonized the actions of H_2O_2 completely. These findings support the hypothesis that an H_2O_2-generating heme protein is involved in the O_2 sensing system regulating gluconeogenic and glycolytic gene expression in response to O_2.

Key words: Phosphoenolpyruvate carboxykinase; Aldolase; Oxygen; Hydrogen peroxide; Metabolic zonation

1. Introduction

The knowledge of O_2 sensing systems in mammals is still rather limited. In principle an O_2 sensing system should consist of the sensor proper, which binds O_2, and a regulator, which interacts with DNA or RNA. In the simplest case the system would consist of a single protein in analogy to the steroid receptor with an input domain, the sensor proper, which senses the O_2 tension via a heme component, and an output domain, the regulator proper, which binds to oxygen-responsive elements in the 5′ or 3′ sequences of genes or to the 3′ end of an mRNA and thus regulates gene expression or mRNA stability in response to O_2. O_2 sensor and regulator may also be separate proteins linked by a chemical reaction: the sensor could be a heme protein located in the plasma membrane with a protein kinase activity similar to the fixL/fixJ system of the root nodule bacterium *Rhizobium meliloti* [1] or with NADPH-oxidase activity producing H_2O_2 as shown for airway chemoreceptors [2]. The regulator protein with its DNA/RNA binding activity would then be phosphorylated or oxidized, respectively, in response to O_2 [3].

In hepatocytes the gene of the major rate generating gluconeogenic enzyme, cytosolic phosphoenolpyruvate carboxykinase (PCK), was induced by glucagon to higher transcription rates, mRNA amounts as well as protein levels under arterial than under venous oxygen tensions, while degradation of PCK protein was not affected by different O_2 concentrations [4–6]. Conversely, the genes for the glycolytic enzymes lactate dehydrogenase A (LDH A), phosphoglycerate kinase 1 (PGK1) and aldolase A (ALD A) as well as the erythropoietin (EPO) gene were induced by hypoxia in Hep3B and HepG2 cells [7,8]. Studies with the O_2 competitive ligand carbon monoxide showed that this was due to a heme protein which belongs to the *b*-type cytochromes [9–11]. Western blots with antibodies against the small subunit (22 kDa) and the cytosolic activation factor (47 kDa) of NADPH oxidase demonstrated the presence of an NADPH oxidase-like heme protein in HepG2 cells [11]. This was further substantiated by the finding that HepG2 cells produced H_2O_2 in response to O_2 and that the hypoxia-induced EPO production could be inhibited by H_2O_2 [12].

It was the aim of the present study to investigate the role of H_2O_2 as possible mediator in the O_2 sensing system of primary hepatocytes which causes the positive modulation by arterial O_2 of the glucagon-dependent activation of the PCK gene and elicits the activation of the ALD A gene by venous O_2. If H_2O_2 were the intracellular messenger for normoxia, it should be produced in response to O_2. In addition exogenously added H_2O_2 should override the O_2 sensor and cause the same high glucagon-dependent induction of PCK mRNA at hypoxia as at normoxia and prevent ALD A induction by hypoxia. Thus H_2O_2 should abolish O_2-dependent differences in the induction of PCK and ALD A. Therefore the production of H_2O_2 in response to the pericellular O_2 and the effects of different concentrations of added H_2O_2 and/or catalase as H_2O_2 scavenger on PCK and ALD A induction were studied in rat hepatocyte cultures. Hepatocytes released H_2O_2 in response to O_2 and exogenous H_2O_2 acted as predicted. Thus it is proposed that an H_2O_2 generating heme protein is involved in the O_2 sensing system regulating gluconeogenic and glycolytic gene expression in response to O_2 as is the case with the EPO gene.

2. Materials and methods

2.1. Chemicals

All chemicals were of reagent grade and purchased from commercial suppliers. Collagenase, digoxigenin-UTP, the digoxigenin nucleic acid detection kit and fetal calf serum were from Boehringer (Mannheim). Medium M199 was from Gibco BRL (Eggenstein). T3 RNA polymerase was from United States Biochemicals. Hormones were

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delivered from Serva (Heidelberg). Guanidinium thiocyanate was purchased from Fluka (Neu-Ulm) and nitrocellulose BA-S 85 from Schleicher and Schüll (Dassel). Hyperfilm and [^{14}C]leucine were supplied by Amersham Buchler (Braunschweig). All other chemicals were from Sigma (Taufkirchen).

2.2. Animals

Male Wistar rats (200–260 g, Winkelmann, Borchen) were kept on a 12 h day/night rhythm (light from 7 a.m. to 7 p.m.) with free access to water and food. Rats were anesthetized with pentobarbital (60 mg/kg body weight) prior to preparation of hepatocytes between 8 a.m. and 9 a.m.

2.3. Cell culture and induction 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 (by vol.) in medium M199 containing 1 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 to 24 h at 16% O_2 (mimicking arterial O_2 tensions). At 24 h induction of PCK was started by adding fresh M199 with 0.1 nM glucagon and when indicated 50 μM H_2O_2 and/or 100 μg/ml catalase each at 16% O_2 or at 8% O_2 (mimicking venous O_2 tensions). The O_2 values take into account the O_2 diffusion gradient from the media surface to the cells [4].

2.4. Measurement of extracellular release of H_2O_2

H_2O_2 production was measured according to Ruch et al. [13] and is based on the conversion of luminol by H_2O_2 in the presence of peroxidase. After 24 h of culture the media were completely removed and the cells were washed three times with 0.9% NaCl. Culture media were then replaced by the same volume of a Krebs-Ringer buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.4 mM $NaHCO_3$). Cells were then further cultured under arterial and venous O_2 tensions. Every 2 h H_2O_2 was determined in Krebs-Ringer buffer containing 10 μg/ml peroxidase and 10 μg/ml luminol in a luminometer (Berthold, Germany). Krebs-Ringer buffer alone served as an independent control. The H_2O_2 concentrations used to establish standard curves were prepared by dilution of a 30% solution of H_2O_2 in Krebs-Ringer buffer.

2.5. RNA preparation and Northern analysis

Total RNA was prepared from 3×10^6 cells as described [14]. 15 μg RNA was denatured by formaldehyde and used in a typical Northern experiment. Digoxigenin (DIG)-labelled antisense ALD A and PCK RNA served as hybridization probes; they were generated by in vitro transcription from pBS-ALDA, pBS-PCK 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 of the DIG-nucleic acid detection kit (Boehringer Mannheim). Detection of hybrids was performed as described before [6]. Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen).

2.6. 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 [^{14}C]leucine into TCA precipitable protein [6]. Cell morphology was controlled by light microscopy.

3. Results

In primary rat hepatocytes the glucagon-dependent activation of the PCK gene as well as the expression of the ALD A gene were modified by O_2. Heme proteins with H_2O_2 producing oxidase were proposed to be involved in the O_2 sensing system regulating these processes. To test for the involvement of H_2O_2 as possible mediator in the O_2 sensing of primary hepatocytes the H_2O_2 release in response to the pericellular O_2 was measured and cells were treated with H_2O_2 and/or catalase to override the sensing system.

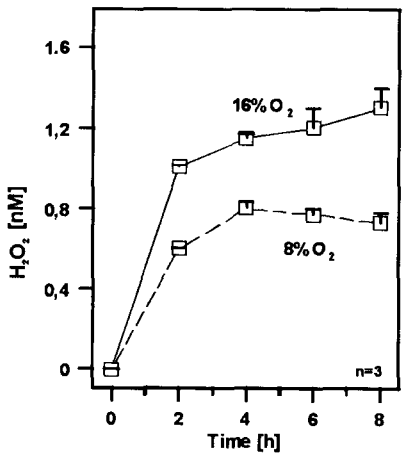

Fig. 1. O_2-dependent release of H_2O_2 from rat hepatocyte cultures. Primary hepatocytes were cultured for 24 h at arterial O_2. The medium was then replaced by a Krebs-Ringer buffer. The cells were further cultured under either arterial or venous O_2 tensions. Released H_2O_2 was determined at the indicated time points by a chemiluminescence assay. Values are means ± S.E.M. of 3 independent culture experiments.

3.1. Release of H_2O_2 in response to the pericellular O_2 tension

In primary rat hepatocytes the pericellular O_2 tension influenced the production and release of H_2O_2. With subsequent culture under arterial and venous O_2 tensions the hepatocytes released H_2O_2 into the medium in correlation with the O_2 tension (Fig. 1). After 4 h nearly steady-state levels were reached; the external concentration was about 1.2 nM under arterial O_2 and about 0.8 nM under venous O_2.

3.2. Optimal concentrations of H_2O_2 and catalase, and cell viability

The basal levels of PCK mRNA were not influenced and [^{14}C]leucine incorporation into total TCA-precipitable protein proceeded unaltered up to an H_2O_2 concentration of 200 μM. Also, the induction by glucagon of PCK mRNA was not negatively impaired by H_2O_2 concentrations up to 200 μM; on the contrary, induction was even stimulated by about 30% after treatment of cells with 50 μM and 100 μM H_2O_2 (Fig. 2). Thus, treatment with H_2O_2 up to 200 μM had no adverse effects on cell viability. The stimulatory H_2O_2 concentration of 50 μM was chosen for further experiments. The optimal concentration of catalase was deduced from a previous study with HepG2 cells [12]: exposure of these cells to 100 μg/ml catalase did not reduce cell viability.

3.3. Enhancement by H_2O_2 of the glucagon-dependent induction of PCK mRNA and inhibition of the venous O_2-dependent induction of ALD A mRNA

In 24 h rat hepatocyte cultures glucagon induced PCK mRNA to a maximum within 2 h; thereafter PCK mRNA declined again in line with previous studies [5,6,10] (not shown). Glucagon elevated PCK mRNA by about 5.5-fold (= 100% induction) at arterial pO_2 and about 3.6-fold (= 65% induction) at venous pO_2 (Fig. 3). The basal values of PCK mRNA were not changed during the 2 h period at the different pO_2 values (not shown). 50 μM H_2O_2 enhanced the glucagon-dependent induction of PCK mRNA 6.5-fold (= 130% induction), at arterial and about 6.0-fold (= 118%

induction) at venous oxygen tensions. The small difference between the values at arterial and venous pO_2 (130% vs. 118%) was no longer significant (Fig. 3). The basal PCK mRNA levels were not influenced by treatment with H_2O_2 (Fig. 2).

In the same 24 h rat hepatocyte cultures ALD A mRNA levels were not altered significantly within 2 h under basal conditions at arterial pO_2; however they were enhanced about 1.7-fold (=100% induction) at venous pO_2. 50 μM H_2O_2 suppressed ALD mRNA induction by hypoxia (Fig. 3).

Thus, H_2O_2 simulated arterial or even slightly hyperoxic conditions. It enhanced the glucagon-dependent induction of PCK mRNA at venous pO_2 to values higher than those obtained at arterial pO_2 in the absence of H_2O_2 and it inhibited the induction of ALD A mRNA at venous pO_2.

3.4. Specificity of the H_2O_2 actions

The specificity of the H_2O_2 effects could best be studied at venous pO_2. It appeared possible that the added H_2O_2 could be converted to O_2 and H_2O by endogenous catalase and that it could therefore act by simply increasing the intracellular pO_2. This possibility was tested by adding catalase to the culture media in the presence and absence of H_2O_2. Catalase would then split the added H_2O_2 to O_2 and H_2O and increase extracellular pO_2 and thus also intracellular pO_2. At venous pO_2 catalase alone did not alter the induction of PCK mRNA by glucagon; it enhanced the induction of ALD A mRNA by hypoxia slightly and insignificantly (Table 1). Catalase added together with H_2O_2 abolished the increase in the glucagon-dependent induction of PCK mRNA and the suppression of the hypoxia-elicited induction of ALD mRNA by H_2O_2 (Table 1). It can therefore be concluded that H_2O_2 acted directly as such and not indirectly by increasing the extracellular and then the intracellular pO_2.

4. Discussion

The present investigation has shown that primary rat hepatocytes produced H_2O_2 in response to the pericellular pO_2. Furthermore it has been demonstrated that the activation of the PCK gene by glucagon was enhanced by treatment with H_2O_2 to the same levels at arterial and venous pO_2. The normal modulation by O_2 of this induction was lost after addition of H_2O_2 which should mimic high arterial O_2 tensions. Conversely H_2O_2 inhibited the low O_2-dependent induction of the ALD A gene. Since H_2O_2 could be produced by a heme containing oxidase these findings substantiate the proposal that H_2O_2 could function as mediator in the O_2 sensing system modulating the glucagon-dependent induction of the PCK gene as well as the low O_2 -dependent induction of the ALD A gene.

Table 1
Enhancement of the glucagon-dependent induction of PCK mRNA and inhibition of the hypoxia-elicited induction of ALD A mRNA by H_2O_2 in primary rat hepatocytes under venous pO_2

Treatment	PCK mRNA induction (%)	ALD A mRNA induction (%)
none	65 ± 5	100 ± 18
+H_2O_2 (50 μM)	118 ± 9*	28 ± 15*
+Catalase (100 μg/ml)	63 ± 5	142 ± 20
+H_2O_2+Catalase	63 ± 9	121 ± 12

The experiments were performed as described in Fig. 3. In the control experiments without H_2O_2 and/or catalase the maximal PCK mRNA induction under arterial pO_2 and ALD A mRNA induction under venous pO_2 was set equal to 100%. Induction is the difference between induced and non-induced mRNA levels. Values measured after 2 h of induction are means ± S.E.M. of 5 (PCK) and 3 (ALD A) individual experiments.
*$P < 0.05$, Student's t-test for paired values (control vs. treatment).

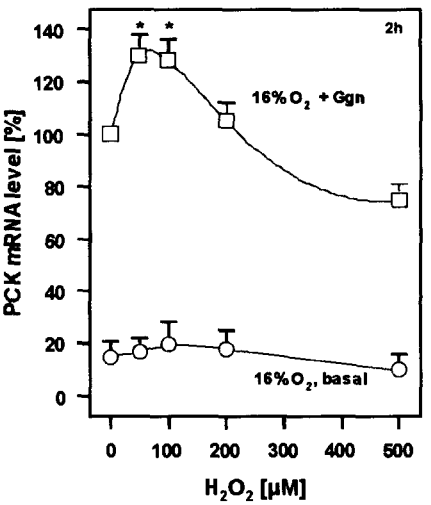

Fig. 2. Glucagon-dependent induction of PCK mRNA in primary rat hepatocytes cultured under arterial pO_2. Increase by H_2O_2. Hepatocytes were cultured for 24 h at arterial O_2. After a medium change PCK was induced with 0.1 nM glucagon in the presence of the indicated concentrations of H_2O_2 for 2 h under arterial O_2 tensions. In glucagon-treated cells the induction of PCK mRNA was enhanced by H_2O_2, whereas basal levels in untreated cells were not changed. In each control experiment with 0.1 nM glucagon but without H_2O_2 the maximally induced PCK mRNA level was set equal to 100%. Values are means ± S.E.M. of 3 independent culture experiments. Student's t-test for paired values: *, significant difference to values without H_2O_2, $P < 0.05$.

4.1. H_2O_2 as an intracellular mediator

4.1.1. H_2O_2 in insulin action on adipocytes. H_2O_2 in the range of 500 μM mimicked the effect of insulin on glucose transport and oxidation, incorporation of glucose into lipid, inhibition of hormone-stimulated lipolysis and stimulation of pyruvate dehydrogenase in isolated adipocytes [15]. In a concentration of 2–3 mM H_2O_2 was shown to activate tyrosine phosphorylation of the insulin receptor and the insulin receptor kinase in intact adipocytes, H-35, and CHO cells [16,17]. In line with these findings insulin stimulated peroxide production through the activation of membrane-bound NADPH oxidase in adipocytes [18].

4.1.2. H_2O_2 in cytokine action in leucocytes. Cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) induced H_2O_2 production in leukocytes (burst reaction) during defense and inflammatory reactions via activation of NADPH oxidase. H_2O_2 could then in turn activate the eukaryotic transcription factor B (NFB) to alter gene expression in the immune response. This was shown by treatment of Jurkat T cells with TNF and H_2O_2. 100 μM H_2O_2 mimicked the effect of TNF in the activation of NFB [19,20].

4.1.3. H_2O_2 in O_2 actions on vascular smooth muscle and hepatoma cells. In pulmonary arteries H_2O_2 induced vasodi-

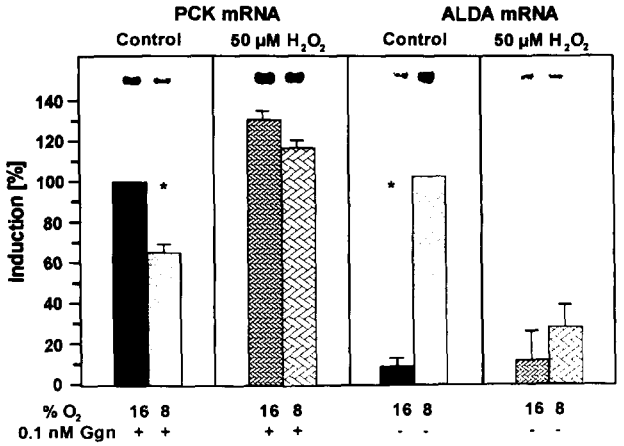

Fig. 3. Modulation by O_2 of the glucagon-dependent increase in PCK mRNA and of the basal ALD A mRNA levels in rat hepatocyte cultures. Simulation of arterial O_2 by H_2O_2. Primary hepatocytes were cultured for 24 h at arterial O_2. After a medium change PCK mRNA was induced with 0.1 nM glucagon. Cells were then further cultured either without or with 50 μM H_2O_2 under arterial and venous O_2 tensions. PCK mRNA and ALD A mRNA were determined after 2 h. In each control experiment without H_2O_2 the maximal PCK mRNA induction under arterial pO_2 and ALD A mRNA induction under venous pO_2 was set equal to 100%. Induction is the difference between induced and non-induced mRNA levels. Values are means ± S.E.M. of 5 (PCK) and 3 (ALD A) independent experiments. Student's *t*-test for paired values: *, significant difference between arterial and venous pO_2, $P < 0.05$.

latation whereas under hypoxia, when H_2O_2 production was low, vasoconstriction was observed [21]. The recent finding that the hypoxia-induced EPO production in HepG2 cells is inhibited by H_2O_2 [12] supports the idea that H_2O_2 could be a mediator of O_2 signals.

4.1.4. H_2O_2 in O_2 actions on hepatocytes. In this study concentrations up to 100 μM enhanced the glucagon-dependent induction of PCK mRNA, whereas inhibitory effects were observed with concentrations higher than 100 μM (Fig. 2). These were not unspecific effects of H_2O_2, since catalase completely inhibited the action of H_2O_2 within the induction period (Table 1). In liver cells the normal intracellular concentration of H_2O_2 was found to be in the sub-micromolar range, whereas other tissues such as the eye lens reached up to 25 μM [22]. The extracellular H_2O_2 concentrations of 50 μM, which mimicked arterial O_2 in this study, did not exert severe oxidative stress, since the oxidative stress responsive gene of heme oxygenase 1 was activated in the same hepatocyte cultures starting with 100 μM H_2O_2 [23]. The results of this study suggest also that the O_2-sensing system in HepG2 cells, which controls EPO production, may be similar to that in hepatocytes which regulates the modulation by O_2 of the glucagon-induced PCK gene expression and the low O_2-dependent expression of the ALD A gene.

Thus, H_2O_2 may play a role in the mediation of insulin action, in the regulation of gene expression during defense reactions and as shown here in the transduction of the O_2 signal into the cell.

4.2. Involvement of heme proteins with H_2O_2 producing oxidase functions in O_2 sensing

4.2.1. Cyanide-insensitive NADPH oxidase. Measurement of absorbance maxima typical for a *b*-type cytochrome, the presence of several components of the NADPH oxidase complex and the detection of H_2O_2 by rhodamine fluorescence suggested that HepG2 cells posses a cyanide-insensitive electron-transfer chain similar to the NADPH oxidase in neutrophils [11] and that they produce H_2O_2 depending on the O_2 tensions. In pulmonary neuroepithelial bodies histochemically stained with an antibody recognizing the p91 polypeptide of the heme-linked NADPH oxidase, H_2O_2 production during O_2 sensing was reported [2].

4.2.2. Various H_2O_2-generating heme proteins. It was shown that a H_2O_2-generating heme protein might act as possible O_2 sensor in carotid body preparations [24]. Spectral analyses and the fluorescence microscopic demonstration of H_2O_2 production revealed an H_2O_2-generating heme protein as a possible O_2 sensor [24].

The hypothesis that an NADPH-like oxidase is part of the O_2 sensor is in line with results from this and earlier studies on the regulation of EPO and PCK induction, which had shown the participation of a heme protein [6,9,10]: the hypoxia-induced EPO production was modulated by inducers and inhibitors of a *b*-type cytochrome, the P-450 system [25], and it was sensitive to O_2 but not cyanide [11] or dinitrophenol [10], which excludes respiratory chain type-*b* cytochromes. Moreover, the hypoxia-induced increases in EPO mRNA [12] and ALD A mRNA were inhibited and the glucagon-dependent induction of PCK mRNA was increased by H_2O_2 (Fig. 3).

4.3. Possible mechanism of H_2O_2 action

4.3.1. Short-term regulation by H_2O_2 without gene activation. The blockade of H_2O_2 production in neuroepithelial bodies mimicked the reduction in the K^+ current which is normally seen under hypoxic conditions [2]. In pulmonary arteries H_2O_2 induced relaxation via a catalase-sensitive activation of a guanylate cyclase [21].

4.3.2. Long-term regulation by H_2O_2 via gene expression

H_2O_2 could influence the activation state of transcription factors as shown for NFB, the oxidative stress-responsive transcription factor [20], or activator protein 1 (AP-1) [26], p53 [27], redox factor 1 (Ref-1) [28], hypoxia inducible factor 1 (HIF1) [29] and recently also C/EBP-β [30]. In a recent study it was demonstrated that H_2O_2 could diminish HIF1 binding activity to the 3'-EPO gene enhancer [29].

4.4. Involvement of phosphorylation/dephosphorylation in O_2 sensing

The H_2O_2 effects do not preclude the possibility that phosphorylations/dephosphorylations are not involved in O_2 sensing. It was shown that hypoxia-induced EPO mRNA as well as HIF1 DNA binding to the 3'-EPO gene enhancer was reduced after treatment of Hep3B cells with the protein kinase inhibitor 2-aminopurine [31]. Thus protein phosphorylations and redox mechanisms may play different roles in O_2 sensing and the exact mechanism by which H_2O_2 enhances PCK induction and inhibits the low O_2-dependent ALD A and EPO gene activation remains to be further elucidated.

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