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Identification of argininosuccinate lyase as a hypoxia-responsive gene in rat hepatocytes

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Background/Aims: **The differential oxygenation of periportal and perivenous hepatocytes has been demonstrated as a major determinant in the zonated expression of certain metabolic pathways in the liver. We have searched for novel genes whose expression could be modulated by hypoxia in cultured rat hepatocytes.**
***Methods*: Primary cultures of rat hepatocytes were incubated under normoxic (21% oxygen) or hypoxic (3% oxygen) conditions for 6 h. Differences in gene expression under both conditions were analyzed using the technique of differential display by means of PCR.**
***Results*: We have identified the enzyme argininosuccinate lyase (ASL) as being downregulated by hypoxia. ASL is a cytosolic protein which participates in urea metabolism. ASL expression was time-dependently reduced in hypoxia. Hypoxia modulated the responses of this gene to the two main hormonal signals which induce ASL mRNA: glucocorticoids and cAMP. ASL mRNA levels decreased in response to ATP-reducing agents. $CoCl_2$ mimicked the effect of hypoxia, suggesting the implication of a hemoprotein in this response. Hypoxia did not affect ASL mRNA stability, indicating that this effect occurs at the transcriptional level.**
***Conclusions*: Our observations suggest that differences in oxygen levels across the hepatic parenchyma could participate in the zonated expression of ASL.**

Key words: **Argininosuccinate lyase; Gene expression; Hypoxia; Liver.**

The liver possesses an enormous and diverse catalytic potential which is mainly met by the parenchymal cell In spite of the histological uniformity of this organ, functional differences have been reported for parenchymal cells located in the periportal and the pericentral or perivenous areas, leading to the concept of the metabolic zonation of the liver (1) This zonation of the liver includes most of the key functions of the organ, such as oxidative and carbohydrate metabolism, amino acid metabolism and ammonia detoxification, bile formation, plasma protein synthesis and xenobiotic detoxification (reviewed in 2) Numerous results indicate that zonation of liver tissue is attained by the differential expression of key enzymes involved in the metabolic pathways mentioned above (1,2)

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Many of the biological signals that modulate the gene expression pattern of the distinctly located hepatic cells are generated by a gradient in the concentration of oxygen, hormones, substrates and blood-borne products circulating from the periportal to the perivenous compartments Oxygen tension in the perivenous area is reduced to about half of that found in the periportal area (35 *vs* 65 mmHg), and has been identified as one of the major determinants in the zonal expression of carbohydrate-metabolizing enzymes (2)

Apart from playing a role in the metabolic zonation of the liver, regulation of gene expression by oxygen levels may represent a more general adaptive mechanism of the cell and the organism to low oxygen supply This is evidenced by the nature of the different hypoxia-responsive genes so far identified, which include erythropoietin, vascular endothelial growth factor, aldolase A, tyrosine hydroxylase, the glucose transporter *glut1* gene (3–7) and hypoxia-downregulated genes such as methionine adenosyltransferase (8)

In order to gain more insight into the genetic mechanisms behind this adaptation, we have searched for

genes whose expression might be modulated by hypoxia in cultured rat hepatocytes Our analysis employing the technique of differential display by means of polymerase chain reaction (PCR) (DDPCR) (9) has allowed us now to identify argininosuccinate lyase (EC 4 3 2 1, ASL) as a novel hypoxia-regulated gene, which is downregulated by low oxygen levels ASL is a cytoplasmic enzyme highly expressed in the adult liver, as compared to other tissues, where it plays a role in urea synthesis (10), a metabolic pathway differentially expressed across the periportal and perivenous areas (2) We have also addressed the influence of hypoxia on the hormonal regulation of ASL expression in cultured rat hepatocytes as well as the mechanisms behind this effect

Materials and Methods

Materials

All chemicals were of reagent grade and obtained from commercial suppliers Collagenase, actinomycin D, catalase and restriction enzymes were from Boehringer Mannheim (Mannheim, Germany) Triamcinolone, $CoCl_2$ desferrioxamine forskolin, and menadione were from Sigma (St Louis, MO, USA)

Isolation and culture of rat hepatocytes

Liver parenchymal cells were isolated from male Wistar rats (200 250 g of weight) by collagenase perfusion, as described (8) Cells were plated and cultured as described (8) Cells were maintained at 37°C in a humidified incubator containing 21% oxygen and 5% CO_2 in air (normoxic conditions) Hypoxic conditions were attained by exposure to 3% oxygen and 5% CO_2 with the balance as nitrogen in a humidified Billups-Rothenberg Modular Incubation Chamber (MIC-101) (Del mar, CA, USA) at 37°C (8) Cell viability was measured by trypan blue exclusion, no significant differences were observed between normoxic and hypoxic cultures at any culture time tested

Differential display by means of PCR analysis

DDPCR was performed on total cellular RNA, isolated as described (11), after DNAseI treatment, using oligo(dT) anchored primers with Hieroglyph mRNA Profile Kit (Genomyx Beckman Instruments, Fullerton, CA, USA) as described (9,12) Nucleotide sequence homology search analysis of the EMBL (13) and GeneBank (14) databases were performed using the program FASTA (15)

RNA isolation and Northern blot analysis

Total hepatocyte RNA was isolated by the guanidinium thiocyanate method (16) Electrophoresis of RNA, gel blotting, prehybridization and hybridization of membranes were carried out as described (11,12) The *glut1* cDNA probe was the generous gift of Dr Antonio Zorzano (Universidad de Barcelona, Barcelona, Spain) The probes were labeled with [α-^{32}P] dCTP (Amersham, Little Chalfont, UK) using the Megaprime DNA labeling system (Amersham) Equal loading of the gels was assessed by hybridization with an 18S ribosomal RNA probe Quantitation was performed by scanning densitometry of the X-ray films

When the effect of actinomycin D on the regulation of ASL mRNA levels by hypoxia was studied, hepatocytes were cultured in the presence of 1 μM triamcinolone to induce ASL expression for 10 h then actinomycin D (5 μg/ml) was added and after 1 h cells were further incubated under normoxic or hypoxic conditions for the indicated periods of time

Measurement of ASL activity

ASL activity was measured on hepatocyte homogenates as described by Tomlison & Westall (17) Results are expressed as micromoles of urea formed per hour (units) per milligram of protein Protein content in homogenates was measured as described (18)

Immunoblot analysis

Cultured hepatocytes were lysed as described (11) Total protein was determined in the extracts as described (18) Electrophoresis and electroblotting were carried out as described (12) Immunodetection of the glucocorticoid receptor (GR) was performed using a polyclonal anti-GR antiserum (Santa Cruz Biotechnology Santa Cruz CA, USA) and a horseradish peroxidase-conjugated secondary antibody Blots were developed by enhanced chemoluminescence according to the manufacturer's instructions (Dupont Boston MA, USA)

Statistics

Data are means±SEM of at least four independent experiments performed in duplicate Statistical significance was estimated with Student's *t*-test A p-value of <0.05 was considered significant

Results

Identification of ASL as a hypoxia-responsive gene

DDPCR analysis was carried out on rat hepatocytes cultured for 6 h in normoxia or hypoxia Several bands were differentially expressed in the two culture conditions One of the bands selected for analysis, which was downregulated in the hypoxic cells (Fig 1A) was excised from the gel, amplified and sequenced Sequencing of 0 32 kb of the total 0 6 kb of this clone revealed a 100% identity to rat ASL cDNA (nucleotides 2101 to 2422) (19), and could hybridize a 2 4 kb mRNA in total cellular RNA from hepatocytes (Fig 1A) The band detected using Northern analysis corresponded in size to that reported for the ASL transcript in rat liver (20), and showed a 60% reduction during hypoxia Hybridization with a cDNA probe for the *glut1* gene, a well-known hypoxia-responsive gene which is induced by this condition (7), was performed as a control As shown in Fig 1B, GLUT-1 mRNA showed a sharp increase (6-fold after 6 h of treatment) during hypoxia

We next performed a time course experiment in order to further characterize ASL mRNA response to hypoxia As shown in Fig 1B a reduction in cellular ASL mRNA was detected after 3 h of incubation under hypoxia, and was even more pronounced at 12 h of incubation The concomitant induction of GLUT-1 mRNA is also shown As reported for other genes which are highly expressed in liver (8,21), ASL mRNA levels in normoxia decreased with time in culture, however, the effect of hypoxia was still evident even after longer incubations (up to 17 h, data not shown)

We also measured ASL enzymatic activity in hepatocytes maintained in normoxia and hypoxia Our results indicate that after prolonged exposure to hypoxia (17 h) ASL activity remained unchanged (0 21±0 02 μmol/h/mg of protein in normoxia *vs* 0 19±0 01 μmol/h/mg of protein in hypoxia)

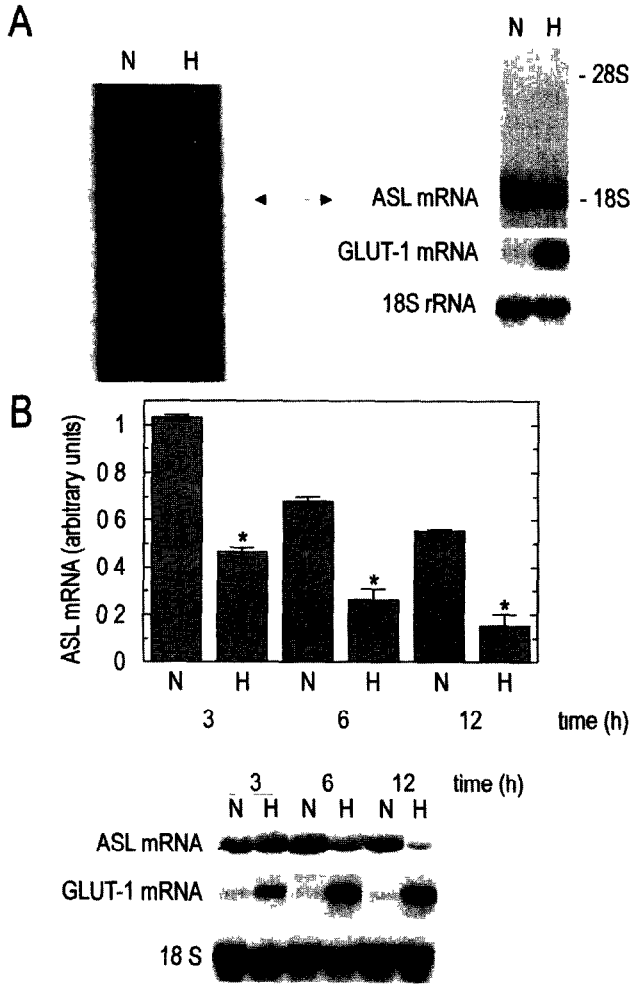

Fig 1 Detection of differential gene expression by hypoxia in isolated rat hepatocytes by differential mRNA display analysis A Sequencing gel electrophoresis of PCR amplified cDNAs performed in duplicates, from normoxic (N) and hypoxic (H) cultures A differentially displayed fragment (arrow) was detected, isolated, sequenced and identified as a 0 6 kb fragment of ASL cDNA The right panel shows a Northern blot analysis of total RNA from normoxic (N) and hypoxic (H) rat hepatocytes performed with ASL cDNA fragment This assay confirmed its differential expression between normoxic and hypoxic cultures Induction of GLUT-1 mRNA expression by hypoxia is also shown Hybridization with a probe for 18 S rRNA was performed as loading control B Time-course of the response of ASL to hypoxia in cultured rat hepatocytes Asterisks indicate $p<0\ 05$ *with respect to normoxic controls Lower panel shows a representative Northern blot The time-dependent induction of GLUT-1 mRNA by hypoxia is also shown Hybridization with a probe for the 18 S rRNA was performed as loading control*

Hypoxia modulates ASL responses to glucocorticoids and cAMP

Glucocorticoids and glucagon have been shown to upregulate ASL mRNA in rat liver and cultured hepatocytes, and their effect is cumulative when added together (10) We have tested whether hypoxia could modulate the effect of both hormones on ASL expression As shown in Fig 2, when hepatocytes were treated for 6 h with triamcinolone (1 μM) or the cAMP generating agent forskolin (10 μM), ASL mRNA levels were induced (6-fold and 5-fold, respectively, over control levels) As described, the effect of both agents was cumulative when used in combination (12-fold induction) When these treatments were performed in hypoxic conditions the effects of triamcinolone and forskolin, alone or in combination, were partially prevented In order to test whether hypoxia could compromise the effect of glucocorticoids on ASL expression through the impairment of glucocorticoid receptor (GR) expression, we analyzed the levels of GR in hepatocytes kept under normoxic or hypoxic conditions for 6 h in culture medium without triamcinolone As shown in Fig 3, GR protein levels were upregulated in hypoxia

Hypoxia sensing and signaling in ASL mRNA regulation

Inhibition of oxidative phosphorylation and reduction of ATP levels are observed in hepatocytes exposed to

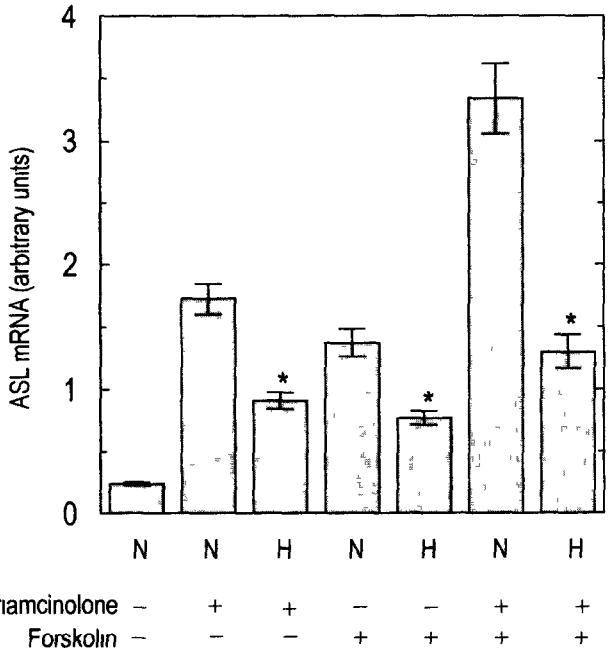

Fig 2 Modulation by hypoxia of ASL response to glucocorticoids and the cAMP inducing agent forskolin Hepatocytes were maintained in normoxic (N) or hypoxic (H) conditions for 6 h in the presence or absence of triamcinolone (1 μM), forskolin (10 μM) or both, and then ASL mRNA was quantitated by Northern blotting Asterisks indicate $p<0\ 05$ *with respect to normoxic controls*

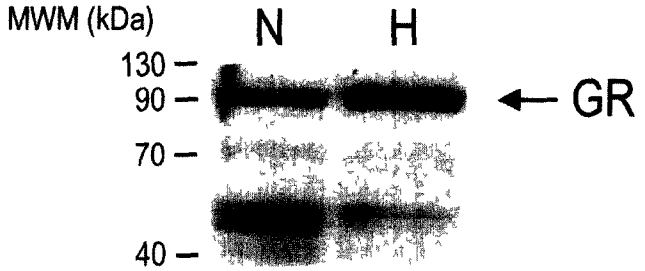

Fig 3 Expression of glucocorticoid receptor (GR) in rat hepatocytes kept in normoxic or hypoxic conditions for 6 h as determined by Western blotting The migration of the molecular weight markers is indicated (MWM) A representative blot is shown

hypoxia (8,22) Some responses to this condition could be the consequence of decreased cellular ATP concentration Thus we wanted to know whether the observed changes in ASL mRNA levels could be related to the inhibition of oxidative phosphorylation For this purpose hepatocytes, cultured in the presence of 1 μM triamcinolone, were treated independently with two inhibitors of mitochondrial respiration, azide and dinitrophenol (DNP), at concentrations previously demonstrated to reduce ATP levels in cultured hepatocytes (8) As shown in Fig 4, treatment for 4 h with either azide or DNP resulted in a significant reduction in ASL mRNA levels (65% and 50% reduction, respectively) and the concomitant induction of GLUT-1 mRNA as described (23)

Studies performed on the regulation of different genes by hypoxia indicate that this condition is primarily sensed by a distinct mechanism, which is independent of changes in ATP levels (3,24) Although the precise nature of the mammalian oxygen sensor remains elusive, the ability of certain transition metals, such as Co^{2+}, to mimic the effect of hypoxia on several hypoxia-responding genes suggests the implication of a heme-based sensor (3,24) We have tested this hypothesis on the expression of ASL mRNA in hepatocytes cultured in the presence of 1 μM triamcinolone When cells were treated for 4 h with 50 μM $CoCl_2$, a concentration previously shown to be non-toxic for rat hepatocyte cultures (25), a reduction in ASL mRNA levels was observed (Fig 4) The concomitant induction of GLUT-1 mRNA in cells treated with $CoCl_2$ was consistent with previous reports (23)

Several lines of evidence have shown that changes in the intracellular levels of reactive oxygen species (ROS) occur during hypoxia, and that ROS could be implicated in the signaling of the hypoxic stimulus (3,24,26,27) We have tested whether alterations in the cellular ROS levels could affect hepatocyte ASL mRNA contents Treatment of cultured hepatocytes under normoxic conditions with the H_2O_2 degrading enzyme catalase (2000 units/ml for 6 h) did not affect ASL mRNA levels (not shown) Similarly the addition of desferrioxamine (DFO) (180 μM for 6 h), an iron chelator capable of preventing hydroxyl radical production (28), resulted in no changes in ASL mRNA levels, either in normoxia or hypoxia (not shown) With similar results the opposite intervention, namely the induction of intracellular H_2O_2 production by treatment with menadione at concentrations which did not compromise ATP levels (50 μM for 6 h) (29,30) produced no changes in ASL expression (not shown)

Mechanism of hypoxia downregulation of ASL mRNA levels

Hypoxia control of gene expression can be accomplished at the transcriptional and postranscriptional levels (24) In order to study the mechanisms involved in hypoxia-mediated downregulation of ASL expression, we measured ASL mRNA levels in normoxic and hypoxic cultures of hepatocytes in the presence or absence of the transcriptional inhibitor actinomycin D (5 μg/ml) As shown in Fig 5A, in the absence of actinomycin D, hypoxia induced the expected time-dependent reduction in ASL mRNA levels However, when the same experiment was performed in the presence of actinomycin D, the effect of hypoxia was not observed (Fig 5B) After 7 h of actinomycin D treatment, ASL mRNA levels in both normoxic and hypoxic cultures

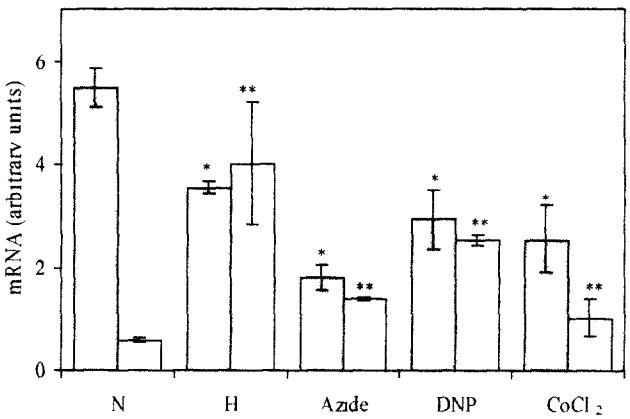

Fig 4 Expression of ASL (closed bars) and GLUT-1 (open bars) mRNAs in rat hepatocytes kept for 4 h in normoxia (N) hypoxia (H) or in normoxic conditions plus 2 mM azide, 50 μM DNP or 50 μM $CoCl_2$ One asterisk indicates $p<0.05$ with respect to the ASL mRNA value in normoxia, two asterisks indicate $p<0.05$ with respect to the GLUT-1 mRNA value in normoxia

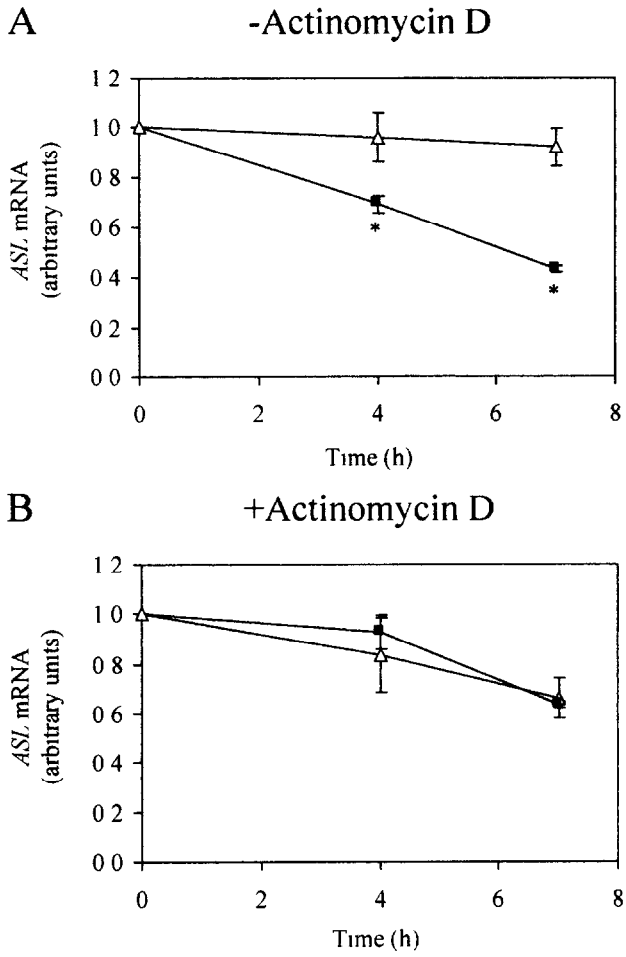

Fig 5 Rat hepatocytes were incubated in normoxic (open triangles) or hypoxic (closed squares) conditions in the absence (A) or presence (B) of 5 μg/ml of the transcriptional inhibitor actinomycin D ASL mRNA levels were quantitated by Northern blotting at different time points Asterisks indicate $p<0\ 05$ *with respect to normoxic values*

were similarly reduced to 60% of the levels found at $t=0$ (Fig 5B), indicating that ASL mRNA decay was not affected by hypoxia

Discussion

Searching for genes whose expression could be modulated in rat hepatocytes by hypoxia we have identified an mRNA corresponding to the urea cycle enzyme ASL as being downregulated by low oxygen levels ASL is the fourth enzyme of the urea cycle and, although it is not a rate-controlling enzyme, plays a role in ammonia detoxification and in arginine production (31) ASL expression in the liver is not homogeneous, *in situ* hybridization has shown that the mRNA for ASL is present only in the periportal region of the hepatic parenchyma (32) We have observed that rat hepatocytes kept under hypoxia display reduced ASL mRNA steady-state levels ASL mRNA downregulation seems to be specific and not attributable to a general derangement of cellular metabolism by hypoxia Cellular viability was not compromised under our experimental conditions Furthermore, we could detect the concomitant and strong induction of the glucose transporter *glut1*, a well-characterized hypoxia-responsive gene (7,23,33), indicating that the transcriptional machinery of the cell was operative

In agreement with the remarkably long half-life of the urea cycle enzymes, including ASL, reported to be in the range of 3 to 9 days (34,35), we did not detect a reduction in ASL enzymatic activity in hypoxia in our model of cultured rat hepatocytes The rate of degradation of a protein can only be determined if steady-state conditions can be maintained for a period at least 2–3 times longer than the expected half-life, and this was not possible in our experimental model Our observation suggests that chronic differences in oxygen availability, such as those observed across the hepatic parenchyma, rather than acute hypoxia, are more likely to modulate ASL expression

ASL expression in liver is under hormonal control, this is mediated by the positive effects of glucagon and glucocorticoids, which display a cumulative effect when added in combination (10,36) We have examined whether hypoxia could modulate the hormonal-dependent induction of ASL expression Our results indicate that hypoxia was capable of counteracting the effects of both the glucocorticoid triamcinolone and the cAMP inducing agent forskolin, when added alone or in combination We have addressed the possibility of an unspecific interference of hypoxia with the cellular responses to glucocorticoids through the impairment of GR expression Conversely, our results show that GR levels in hepatocytes kept in hypoxic conditions were upregulated This is, to our knowledge, the first description of GR induction by hypoxia in hepatic cells, although a similar effect had already been reported in cultured human renal cortex epithelial cells (37) Regarding cAMP signaling in hypoxia, protein kinase A activity has been shown to be decreased in PC12 cells in hypoxia, however, this effect was observed after longer incubation times than those in which we observed ASL mRNA downregulation by hypoxia in the presence of forskolin (38) These observations suggest that the different oxygen tensions present across the hepatic parenchyma could modulate ASL responses to the two main hormones known to regulate its expression

Sensing oxygen levels in bacteria and yeast have been well characterized In these organisms the impli-

cation of heme proteins is central to this process (24) In mammalian cells a similar system is thought to participate in oxygen sensing This has been demonstrated by several lines of evidence, which include the ability of certain transition metals to mimic the effect of hypoxia on gene expression (3) Transition metals such as Co^{2+} could substitute for iron in the heme proteins and lock the heme group in the "deoxy" conformation Mimicry of hypoxia by $CoCl_2$ has been reported for other hepatic genes which are downregulated by this condition, such as phosphoenolpyruvate carboxykinase (PCK-1) (25) and methionine adenosyltransferase (8) Our observation of ASL mRNA downregulation by $CoCl_2$ treatment suggests that a heme-based sensor could participate in this response

One of the major consequences of hypoxia is the inhibition of oxidative phosphorylation and the concomitant decrease in mitochondrial ATP production For some hypoxia-responsive genes, such as erythropoietin, their response to this condition is not dependent on the limitation of mitochondrial metabolism (39) However, other genes modulated by hypoxia are responsive to both the reduction in oxygen levels *per se* and the attendant inhibition of oxidative phosphorylation The best-characterized example of this dual control by hypoxia is the expression of the glucose transporter GLUT-1 (23,33) Our data obtained in primary cultured hepatocytes confirm the previous observations made for *glut1* expression in transformed or immortalized cell lines, and suggest that ASL could participate in this dual mechanism for hypoxia sensing

Hypoxia signaling has been linked to changes in intracellular ROS levels Enhanced and diminished ROS production have been reported to occur during hypoxia, and both conditions have been proposed to convey signals to the cell nucleus capable of modulating gene expression (40) In our hands ASL mRNA levels were not sensitive to any modification in cellular ROS Perhaps other signaling systems such as protein phosphorylation/dephosphorylation, which are also modulated by hypoxia (41–43), could be implicated in the regulation of ASL expression, however, this remains to be examined

Gene transcription and mRNA stability can be affected by oxygen concentration (24) Our data show that hypoxia regulates ASL expression at the transcriptional level This is demonstrated by the lack of effect of hypoxia on ASL mRNA levels when cellular transcription was blocked by actinomycin D treatment Consequently, the decay of ASL mRNA in hepatocytes treated with actinomycin D was the same under normoxic and hypoxic conditions It is known that induction of ASL expression by glucocorticoids and cAMP in hepatocytes is mainly achieved at the transcriptional level (10,44) The ability of hypoxia to inhibit the effect of these agents strongly suggests that this condition is probably acting at the level of ASL gene transcription In this regard, it has recently been reported that hypoxia inhibition of glucagon-induced PCK1 expression in rat hepatocytes is mediated at the promoter level through the interaction of two putative cAMP-responsive elements (45)

In summary, our results show that the expression of ASL can be downregulated by low oxygen levels in cultured rat hepatocytes We have shown that hypoxia can limit the response of this gene to the two hormonal signals which are central to the regulation of this gene Hence, this cellular response of ASL to reduced oxygen availability could participate in the periportal expression of this enzyme

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