Oxygen as a Regulator of Serine Dehydratase (SerDH) Gene Expression.

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An oxygen gradient is formed in the liver due to the unidirectional bloodflow from the portal vein and hepatic artery to the central vein and due to the oxygen-consuming metabolic processes of the cells along the sinusoid. This gradient appears to be one of the major factors responsible for differential expression of a number of genes between periportal and perivenous zones of liver sinusoid [1]. It has been shown that promoters of most genes induced by hypoxia contain a specific O₂ responsive sequence, named the hypoxia response element (HRE) [2]. This sequence can be bound by several related transcription factors known as hypoxia-inducible factors (HIF), among which HIF-1 is studied the most. HIF-1 is a dimer of HIF-1α and HIF-1β, both belonging to the basic helix-loop-helix (bHLH) PAS (Per-ARNT-Sim) pathway.

Introduction. An oxygen gradient is formed in the liver due to the unidirectional bloodflow. Oxygen partial tension (free concentration) equals about 60-65 mm Hg in the periportal blood and falls to about 30-35 mm Hg in the perivenous blood. This gradient in O₂ tension appears to play a key role in the differential expression of a number of genes between the more aerobic periportal zone and less aerobic perivenous zone of liver sinusoid [1].

It has been shown that promoters of most genes induced by hypoxia contain a specific O₂ responsive sequence, named the hypoxia response element (HRE) [2]. This sequence can be bound by several related transcription factors known as hypoxia-inducible factors (HIF), among which HIF-1 is studied the most. HIF-1 is a dimer of HIF-1α and HIF-1β, both belonging to the basic helix-loop-helix (bHLH) PAS (Per-ARNT-Sim) pathway.

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transcription factor family. While HIF-1β was found to be a constitutional protein, HIF-1α protein levels were oxygen-dependent. Although hypoxia may have insignificant effect on the HIF-1α mRNA expression, the major regulation appears to occur posttranslationally on the level of protein stabilization. Under normoxia two proline residues (P402 and P564) within the O2-dependent degradation domain of HIF-1α are subject to hydroxylation by a new family of prolyl hydroxylases. The hydroxylation enables the binding of the von Hippel-Lindau (VHL) tumor suppressor protein, a component of an E3 ubiquitin ligase complex that targets the HIFα-subunits for degradation by the ubiquitin-proteasome pathway.

While the molecular mechanisms of the hypoxia-dependent gene regulation are well characterized, the mechanisms, regulatory transcription factors, and the DNA responsive elements required for normoxia-dependent gene induction are poorly known [3-5]. It was the aim of the present study to characterize the oxygen-dependent expression of serine dehydratase (SerDH) gene, one of the genes upregulated in the more aerobic periportal zone as compared to the perivenous zone of liver sinusoid, and to identify promoter elements responsible for this regulation.

L-serine dehydratase (L-serine ammonia-lyase, EC 4.2.1.13) is an enzyme, catalyzing the pyridoxal phosphate-dependent deamination of serine to produce pyruvate. This enzyme catalyzes the conversion of L-threonine to α-ketobutyrate by the same mechanism and is identical to L-threonine dehydratase (EC 4.2.1.16). It is a homodimeric protein having a 327 amino acid subunit with molecular weight of 34 kDa [6]. SerDH is expressed in liver predominantly and to a lesser extent – in kidney. Its expression is activated by glucagon and glucocorticoids and repressed by insulin [7], while oxygen-dependent regulation of SerDH has not been investigated yet.

**Materials and Methods.** The isolation of primary rat hepatocytes from liver was performed at sterile conditions by the method of collagenase perfusion as described in [8]. Hepatocytes were cultured in medium M199 (Gibco, Eggenstein, Germany) in the presence of dexamethasone (10 μM), insulin (1 nM) and (in the course of the first 4 h after plating) 4% fetal calf serum (FCS). HeLa and hepatoma HepG2 cells were cultured in the MEM medium (PAA Laboratories, Austria) containing 10% FCS. The cells were cultured in CO2 incubator Cytoperm 8080 (Heraeus, Hanau, Germany) at 37°C in humidified atmosphere containing 8% O2 (mild hypoxia) or 16% O2 (normoxia), 5% CO2 and 87% or 79% N2, respectively. Taking into account oxygen diffusion through culture medium, oxygen concentrations in CO2 incubator correspond to the physiological oxygen concentrations on the surface of perivenous and periportal hepatocytes [1].

To construct the plasmids pGL3SerDH-2303, pGL3SerDH-2128, pGL3SerDH-937, pGL3SerDH-471, and pGL3PCK-493, the vector pGL3 basic (4818 b.p.) (Promega, Mannheim, Germany), containing the Firefly luciferase (Luc) gene as a reporter to estimate the promoter activity, was used. The corresponding regions of SerDH promoter were cloned in the polylinker of pG13 basic. The plasmids pG13-SerDH-NRE1, pG13-SerDH-NRE2, pG13-SerDH-NRE3, and pG13-SerDH-NRE4 were constructed using the vector pGL3 promoter (Promega), in the polylinker of which the corresponding oligonucleotides were cloned in front of SV40 promoter. The oligonucleotides containing six copies of the corresponding NRE elements were obtained from the NAPS company (Gottingen, Germany) and HPLC purified. Each repeating unit contained 9 b.p. of the corresponding NRE flanked by 3 b.p. at the 5′- and by 2 b.p. at the 3′-ends. The oligonucleotides also contained additional sequences which were necessary for technical reasons, namely, either the SpeI or Smal restriction sites in the middle of the sequence for the identification of positive clones and both SacI and Nhel sites at the ends. The construct pG13-Epo-HRE contained three copies of the HRE from the erythropoietin gene in front of pG13 promoter and was already described [9]. E. coli K 12 DH5α and XL1-blue strains (Stratagene, Heidelberg, Germany) were transformed by electroporation. Plasmid DNA was isolated using JETstar Plasmid Purification System (Genomed, Bad Oeynhausen, Germany).

Primary rat hepatocytes (1·106 cells per 60 mm dish), HeLa and HepG2 cells (confluent cultures on 60 mm dishes) were transfected using calcium phosphate precipitation method with 2.5 μg plasmid DNA, consisting of 500 ng pRL-V40 (Promega) and 2 μg Firefly luciferase reporter gene construct, as described
before [10]. Construct pRL-SV40, expressing Renilla luciferase gene under control of SV40 promoter, was used to control transfection efficiency. In every culture experiment, two dishes were transfected per measured point. After removal of the media (4 h after transfection), cells were cultured at standard conditions without serum. After 24 h, the medium was changed and cells were cultured for another 24 h either under hypoxia or normoxia. Cells were then washed twice with 0.9% NaCl and incubated for 15 min on a rocking platform with 300 μl of passive lysis buffer supplied with the Dual Luciferase Reporter Assay Kit (Promega). The lysate was then scraped from the plates, vortex-mixed, and centrifuged for 2 min. From the supernatant, 20 μl was assayed for Firefly luciferase activity in a luminometer Auto Lumat Plus LB 953 (Berthold, Pforzheim, Germany). After addition of 100 μl of Stop and Glo™ Reagent (Promega), which quenches the activity of Firefly luciferase, the Renilla luciferase activity was recorded again in a luminometer. Luciferase activity presented in figures was calculated as a ratio between intensities of Firefly and Renilla luciferase luminescence (normalized luminescence). Statistical analysis was performed as described in Legends to Figures.

Total RNA from hepatocytes was isolated using isothiocyanate method as described [11]. The detection of mRNA on Northern blots was performed after hybridization of 15 μg total RNA with digoxigenin-labeled SerDH or β-actin specific probes (DIG RNA Labeling Kit, Roche, Mannheim, Germany). Hybridization signals were visualized by chemiluminescence, and the videodensitometer (Biotec-Fischer, Reiskirchen, Germany) was used for quantification of these signals.

Results and Discussion. Induction of SerDH mRNA expression by glucagon and its modulation by oxygen. Primary rat hepatocytes were cultured for 24 h at standard conditions and then treated for 24 h under hypoxia (8% O₂) or normoxia (16% O₂) either with or
without addition of glucagon. In the absence of its inducer – glucagon, SerDH mRNA expression in primary rat hepatocytes was not detectable (Fig.1). It proves that normoxia alone is not a sufficient stimulus to induce SerDH expression. Indeed, the expression of gluconeogenic SerDH enzyme in vivo at standard conditions is undetectable. However, fasting, leading to secretion of glucagon, stimulates SerDH expression [7].

When the cells were treated for 3 h with 1 nM glucagon, SerDH mRNA expression was induced app. 5-fold under normoxia and app. 2-fold under hypoxia. At a glucagon concentration of 10 nM SerDH mRNA levels were further enhanced app. 12-fold under normoxia and app. 5-fold under hypoxia, thus, SerDH mRNA levels under normoxia were again app. 2.5-fold higher than SerDH mRNA levels under hypoxia. These results indicate that regardless of the presence of different glucagon concentrations, SerDH mRNA expression under hypoxia is much lower than under normoxia (Fig.1).

**Localization of the normoxia responsive elements in the promoter of SerDH gene.** To find out what sequences could be involved in the regulation of SerDH gene expression by oxygen, the SerDH promoter sequence was analyzed for similarities with the NRE of the phosphoenolpyruvate carboxykinase-1 (PCK-1) gene 5'-TTAGGTCCAG-3' [4].

Sequence analysis of the rat SerDH promoter revealed that four putative NREs were present within the first 2303 b.p. of the promoter. All four potential normoxia response elements, namely, NRE-1, -2169/-2161, 5'-TGAGGACAG-3', NRE-2, -1904/-1896, 5'-TTATGTGAG-3', NRE-3, -578/-570, 5'-TTAGTCCAG-3', and NRE-4, +38/+46, 5'-CTAGATCAG-3', match the NRE of the PCK-1 gene in 7 out of 9 b.p.
A 2303 b.p. fragment of the 5'-flanking region of rat SerDH gene was cloned in front of the luciferase gene in pGl3-basic to generate pGl3SerDH-2303. In primary rat hepatocytes transfected with pGl3SerDH-2303 Luc activity was induced maximally under normoxia and to only app. 60% under hypoxia (Fig.2).

The Luc activity in cells transfected with pGl3SerDH-2303 and treated with glucagon was not induced compared to the untreated control (unpublished data). These results are in line with other studies since both cAMP-regulatory elements (CRE-1 and CRE-2) of SerDH gene, potentially involved in the glucagon-dependent gene induction, are located app. 3500 b.p. upstream from the transcription initiation site, i.e. they are not present in the construct pGl3SerDH-2303 or the shorter constructs used in our experiments [12]. A CRE-1 located at -3528/-3521 of the SerDH gene is not involved directly in the gene activation by hormones whereas CRE-2 (-3538/-3531), adjacent to CRE-1 and bound by CREB (CRE binding protein), appears to be critical for cAMP and glucagon-dependent induction of SerDH expression [12].

To determine what region of the SerDH promoter could be responsible for the oxygen-dependent regulation of SerDH gene expression, primary rat hepatocytes were transfected with three serially deleted SerDH promoter luciferase gene constructs pGl3SerDH-2128, pGl3SerDH-937, and pGl3SerDH-471 containing the first 2128 b.p., 937 b.p., and 471 b.p. of the SerDH promoter, respectively (Fig.2). Normoxia did not induce Luc activity in the cells transfected with either pGl3SerDH-2128, pGl3SerDH-937 or pGl3SerDH-471 indicating that NRE-2, NRE-3, and NRE-4 did not seem to be involved in the regulation of SerDH gene expression by normoxia.

These results demonstrated that the expression of the -2303 SerDH promoter Luc gene construct was induced by normoxia and the normoxia responsive region of SerDH was localized between -2128 and -2303 b.p. of the SerDH promoter.

**Biological activity of the potential SerDH-specific NREs under heterologous conditions.** Since hypoxia-responsive elements from erythropoietin and other hypoxia-regulated genes could act as transcriptional enhancers when cloned in front of an independent promoter and a reporter gene, the role of potential NREs from SerDH promoter in the normoxia-dependent gene regulation was investigated using these elements as enhancers, regulating expression of the reporter luciferase gene. The 90 b.p. oligonucleotides containing 6 copies either of NRE-1, NRE-2, NRE-3 or NRE-4 from the SerDH promoter were cloned in front of the SV40 promoter and the luciferase gene in pGl3-prom to generate pGl3-SerDH-NRE1, pGl3-SerDH-NRE2, pGl3-SerDH-NRE3, and pGl3-SerDH-NRE4. When primary rat hepatocytes were transfected with these constructs, neither of them was expressed differentially under normoxia or hypoxia. However, in HepG2 cells the construct pGl3-SerDH-NRE2 was activated by normoxia in about 40%, while the others displayed the same levels of Luc activity under normoxia and hypoxia. In HeLa cells the expression of two out of four constructs, pGl3-SerDH-NRE1 and pGl3-SerDH-NRE2, was activated by normoxia in about 30% (Fig.3). Luc activity in the cells transfected with the hypoxia-inducible pGl3-Epo-HRE construct [9] as a control was significantly higher in the cells cultured under hypoxia for all three cell types investigated (Fig.3).

These results indicate that some potential SerDH NREs are more active in immortalized cells. It is well known that intratumoral hypoxia is one of the driving forces in cancer progression and a lot of genes involved in survival, angiogenesis, and metastases formation are induced by hypoxia [13-14]. On the other hand, some pro-oncogenic proteins (e.g. plasminogen activators) are induced by normoxia [15-16]. Therefore, hypothetical activation of factors stimulated by normoxia because of genetic changes in the process of carcinogenesis might activate these genes even under normoxia.

The majority of genes modulated by O₂ have been shown to be induced by hypoxia. Though several genes have been found to be positively modulated by normoxia [1], little is known about possible normoxia regulatory elements as well as corresponding transcription factors mediating this modulation and a general idea has not been presented. In the current study it was shown that SerDH promoter sequences homologous to the previously identified NRE of the PCK-1 gene [4] were involved in the normoxia-dependent gene regula-
tion. On the other hand, in the human glutathione peroxidase (GPX) gene two similar 13 b.p. oxygen responsive elements were found to bind disparate proteins and to confer normoxia-dependent induction of GPX gene expression in human cardiomyocytes [3, 5]. The sequences of the oxygen responsive elements from the GPX gene are not similar to the NRE sequences identified in our experiments. It might be also speculated that higher gene expression under normoxia as shown in our study could be due to an inhibitory effect of HIF-1 under hypoxia. However, HIF-1 usually acts as an activator and the role of HIF-1 as transcriptional inhibitor was demonstrated only for few genes so far [17]. This mechanism does not appear to account for SerDH modulation by oxygen since its promoter contains no complete 8 b.p. HRE in the 5′-flanking region. The ability of NREs, described in our study, to function in various cell types and in different promoters indicates that these promoter elements are not specific for SerDH gene only.

In the present study the O₂-dependent expression of SerDH mRNA and the O₂-dependent regulation of SerDH promoter activity were demonstrated. Both SerDH mRNA and SerDH promoter-driven luciferase reporter gene stimulation by normoxia (16% O₂) as compared to mild hypoxia (8% O₂) was not modulated by glucagon. Furthermore, the respective normoxia responsive elements in the SerDH promoter were identified and characterized.
Кислород в межклеточном пространстве гепатоцитов как регулятор транскрипции гена сериндеядратазы.

Резюме
В связи с однонаправленным движением крови от портальной вены и печеночной артерии к центральной вене и из-за использования кислорода клетками синусоида в печени формируется градиент концентрации кислорода. Его различные концентрации определяют неравномерную экспрессию генов в клетках, расположенных в перипортальной и периверешечной зонах печеночного синусоида. Ген, кодирующий сериндеядратазу (СДГ), является типичным представителем генов, преимущественно экспрессирующихся в гепатоцитах перипортальной зоны синусоида. Нозерн-блот гибридизации и транскрекция первичных гепатоцитов крыс конструированных, содержащих репортерный ген люкуферазы под контролем промотора гена СДГ (–2303 ... +55 п.н.), выявили, что экспрессия СДГ выше при нормоксии (15 % О2), чем при гипоксии (8 % О2). Четыре потенциальных элемента ответа на нормоксию (ОЭН) обнаружены в промоторе гена СДГ (ОЭН-1, ОЭН-2, ОЭН-3 и ОЭН-4). Чтобы установить, какой из этих элементов функционирует в гепатоцитах, клетки трансформировали конструкциями с репортерным геном люкуферазы, находящимся под контролем в разной степени укороченных участков промотора гена СДГ. Транскрипция клеток HeLa и HepG2 конструкциями, содержащими шесть последовательно расположенных копий каждого из потенциальных ОЭН перед промотором SV40 и репортёрным геном люкуферазы, показала, что ОЭН-2 в клетках HeLa и HepG2, а ОЭН-1 в клетках HeLa участвуют в регуляции транскрипции гена СДГ при нормоксии.

Ключевые слова: ген сериндеядратазы, регуляция транскрипции, репортерный ген, люкуфераза, гепатоциты, нормоксия.

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