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Development and hormonal control of thioredoxin and the thioredoxin-reductase system in the rat liver during the perinatal period

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Summary. The development and hormonal regulation of thioredoxin and of the thioredoxin-reductase system were investigated during the perinatal period in rat liver. An immunological procedure was developed in order to quantify thioredoxin in fetal and neonatal hepatocytes. Both immunoreactive thioredoxin and thioredoxin-reductase activity appeared on day 16.5 of pregnancy. The level of immunoreactive thioredoxin increased during the late fetal period, and its level was the same 24 h after birth. Moreover, its development was not subjected to hormonal regulation by corticosteroids and glucagon. In contrast, thioredoxin-reductase activity increased 3 times during the late fetal period and presented a marked increase 24 h after birth. In the absence of glucocorticoids there was no increase in the level of thioredoxin reductase, while administration of hydrocortisone acetate and glucagon to fetuses prematurely evoked its activity. This study suggests that if thioredoxin acts physiologically, this activity is related to the state of reduction of the molecule rather than to the total concentration in the liver.
Key words. Thioredoxin; thioredoxin reductase; perinatal period; liver; pancreatic hormones; corticosteroid.

The late fetal period is marked in the liver by the appearance and the development of metabolic pathways, such as urea formation in ureotelic mammals. The development of two urea-cycle enzymes, mitochondrial carbamyl phosphate synthetase-I (CPS-I; EC 6.3.4.16) and cytosolic argininosuccinate synthetase (ASS; EC 6.3.4.5), and their hormonal control, have been described previously: in vivo corticosteroids stimulate the accumulation of immunoreactive CPS-I in fetal mitochondria in an inactive form which is further activated by the administration of glucagon [1]. Recently, it was demonstrated in our laboratory that reduced thioredoxin is able to activate CPS-I and ASS in vitro [2-4]. The presence of thioredoxin and of the thioredoxin-reductase system in adult rat liver was previously reported [for review, see Holmgren [5]]. Thioredoxin is a small ubiquitous protein (12 kDa) presenting two redox-active half-cystine residues. Oxidized thioredoxin is reduced in vivo in a reaction catalyzed by thioredoxin reductase (EC 1.6.4.5) with NADPH as substrate. Reduced thioredoxin is involved in the reduction of

protein disulfides such as insulin[6], human choriogonadotropin[7] or somatomedins[8]. Thioredoxin also participates in the reduction of ribonucleotides[9] and methionine sulfoxide[10].

It was consequently interesting to specify whether thioredoxin is also involved in the mechanism of activation of CPS-I and ASS in vivo. Therefore, the development of thioredoxin and the thioredoxin-reductase system, and its hormonal regulation, were investigated during the perinatal period, and after treating fetuses with corticosteroids and glucagon in vivo. Immunoreactive thioredoxin was estimated by means of a sensitive immunoassay using monospecific antibodies, and thioredoxin-reductase activity was also measured. The results were compared with adult values. The mediatory role of thioredoxin in the activation of CPS-I and ASS by glucagon is discussed.

Materials and methods

Animals and experimental procedures. Adult female rats (Shermann strain), kept under standard conditions, were mated overnight and recognized as pregnant by a vaginal smear on the next morning. This was designated as day 0.5 of pregnancy. Under these conditions, birth occurs on day 21.5.

Bilateral adrenalectomy of the mother was performed on day 14.5 of pregnancy. On day 18.5 of pregnancy fetuses were hypophysectomized in utero by the method of Jost[11,12]. Some hypophysectomized fetuses were immediately given an i.p. injection of 50 µg hydrocortisone acetate (Roussel, Paris, France). Control fetuses received 50 µl of a 9 $g \cdot l^{-1}$ NaCl solution. Some fetuses of intact mothers received 50 µg hydrocortisone acetate (Roussel, France), alone on day 18.5, or in association with 25 µg glucagon (Novo, Bagsvaerd, Denmark) on day 20.5. Fetuses of the opposite horn in the same animal were treated with 50 µl of NaCl 9 $g \cdot l^{-1}$ and served as controls.

At intervals, the mothers were killed by cervical dislocation and fetuses were immediately removed from the uterus and sacrificed by decapitation. Their livers were quickly excised, blotted and weighed and a homogenate was prepared. Fetal or adult livers were homogenized in 7 volumes of 0.1% N-acetyl,N,N,N-trimethyl ammonium bromide in 0.05 M Tris-HCl buffer pH 7.4 using a glass homogenizer. After centrifugation at 8,000 × g for 15 min at 4 °C aliquots of the supernatant fractions were used for thioredoxin quantitation and for thioredoxin-reductase activity measurement.

Thioredoxin quantitation. Thioredoxin was purified according to the method of Luthman and Holmgren[13] with minor modifications[4]. A rabbit was immunized with a homogenized thioredoxin preparation, according to the following procedure: 180 µg in complete Freund's adjuvant, 360 µg of thioredoxin in incomplete Freund's adjuvant in two injections at two-week intervals and finally 100 µg in two injections at two-week intervals, the last one in a vein of the ear. Blood was collected five days later. IgG was purified by successive ammonium sulfate precipitations. Immunoreactivity and monospecificity of the antiserum and purified IgG have been previously shown[4]. Thioredoxin quantitation is performed by single radial immunodiffusion[14]. The gel contains 20 mM of $EDTA(Na)_2$ and the sample is treated with 0.1 M of dithiothreitol (DTT, 20 min at 20 °C)[4]. This prevents the aggregation of thioredoxin during diffusion.

Thioredoxin reductase assay. Thioredoxin-reductase activity was determined using dithionitrobenzoic acid (DTNB) as chromogenic substrate[15]. Its activity is expressed as nmoles of reduced DTNB formed per min at 25 °C g $liver^{-1}$ mg $protein^{-1}$. Glutathione was removed from enzyme extracts by dialysis overnight against 20 mM Tris-HCl buffer pH 7.5. Validity of the assay was confirmed by the measurement of the K_m of the purified enzyme, and in the homogenate. The values were the same ($K_m = 6.6 \times 10^{-4}$ M).

Protein determination. Protein concentrations were determined by the Lowry procedure[16], using bovine serum albumin as standard.

Statistical analysis. Statistical analysis was performed using Student's t-test. All values are expressed as the mean ± confidence interval with a 95% confidence limit.

Results

Changes in thioredoxin level and thioredoxin-reductase activity during development. Thioredoxin was detected on day 16.5 of pregnancy when there was about 30 µg/g liver (table). Its level increased about 2.4 times during the late fetal period and reached about 70 µg/g liver on day 21.5 of pregnancy. After birth, the thioredoxin level was unchanged and was 70% of the adult value. Thioredoxin reductase activity was detected as soon as day 14.5 of pregnancy and increased about 3 times between days 16.5 and 21.5 of pregnancy (table). The specific enzyme activity increased markedly 24 h after birth and was about 40% of the adult value.

Effects of corticosteroids on thioredoxin and thioredoxin-reductase system during perinatal period. In the absence of corticosteroids, in hypophysectomized fetuses from adrenalectomized mothers, the thioredoxin content of the liver was not modified (fig. 1), but thioredoxin reductase activity was significantly lower. A single administration of hydrocortisone acetate to hypophysectomized fetuses restored enzyme activity to the level found in untreated fetuses. Moreover, treating intact rat fetuses with hydrocortisone acetate on day 18.5 of pregnancy did not change the thioredoxin level three days later (fig. 2), but significantly increased thioredoxin-reductase activity (about 18%). The thioredoxin reductase activity on day 21.5 of pregnancy was lower in control fetuses which

Time-course of changes in thioredoxin level and thioredoxin-reductase activity in the liver during development

Stage	Thioredoxin level µg / g liver	µg / mg proteins	Thioredoxin-reductase activity nmoles of reduced DTNB / g liver	nmoles of reduced DTNB / mg proteins
Day 14.5	n.d. (2)	n.d. (2)	52.8 (2)	0.64 (2)
Day 15.5	n.d. (3)	n.d. (3)	73.2 ± 15.7 (3)	1.04 ± 0.10 (3)
Day 16.5	29.3 ± 4.0 (5)	0.39 ± 0.06 (5)	136.3 ± 7.6 (7)	1.81 ± 0.07 (7)
Day 17.5	44.8 ± 7.9 (4)	0.61 ± 0.09 (4)	212.6 ± 3.3 (8)	2.93 ± 0.29 (8)
Day 18.5	49.6 ± 3.2 (5)	0.66 ± 0.07 (5)	246.3 ± 7.0 (7)	3.27 ± 0.29 (7)
Day 19.5	50.3 ± 5.4 (5)	0.68 ± 0.06 (5)	288.6 ± 16.0 (7)	3.89 ± 0.31 (7)
Day 20.5	53.3 ± 3.0 (8)	0.75 ± 0.10 (8)	323.4 ± 13.1 (7)	4.54 ± 0.51 (7)
Day 21.5	69.3 ± 6.0 (6)	0.86 ± 0.09 (6)	382.7 ± 8.2 (8)[b]	4.83 ± 0.41 (8)
New born	70.8 ± 3.4 (5)[a]	0.71 ± 0.06 (5)	530.0 ± 9.4 (10)[b, c]	5.18 ± 0.24 (10)
Adult	103.1 ± 7.5 (8)[a]	1.01 ± 0.11 (8)	1304.4 ± 25.1 (8)[c]	12.71 ± 0.77 (8)

Each assay is performed on pooled fetal, neonatal or adult livers. Values shown are the mean ± confidence interval. The number of assays is indicated in parentheses.
[a, b, c]: Statistically different (p = 0.05). n.d.: non-detectable.

received NaCl solution on day 18.5 (fig. 1) than in untreated fetuses (table).

Effects of glucagon in association with hydrocortisone acetate on thioredoxin and the thioredoxin-reductase system. A single administration of glucagon on day 20.5 of pregnancy in fetuses which had received hydrocortisone acetate on day 18.5 of pregnancy increased thioredoxin-reductase activity about 26% without modifying thioredoxin level (fig. 2).

Discussion

This work describes the development of thioredoxin-reductase activity and thioredoxin level during the perinatal period and shows the effects of corticosteroid hormones and glucagon. We have developed a sensitive immunoassay able to detect immunoreactive thioredoxin during the perinatal period. Our values are consistent with those previously reported by Luthman and Holmgren[13] who detected about 10 mg of thioredoxin per 100 g of adult rat liver. Our results show that the developmental time-course and the regulation of the levels of thioredoxin and thioredoxin-reductase activity are quite different.

The accumulation of immunoreactive thioredoxin during the late fetal period appears to be associated with the increase in the percentage of the liver volume that is occupied by hepatocytes[17]. Around birth, the thioredoxin level remained stable, and it probably increased only slightly to adulthood. On the contrary, thioredoxin-reductase activity, which alters the reduced state of thiore-

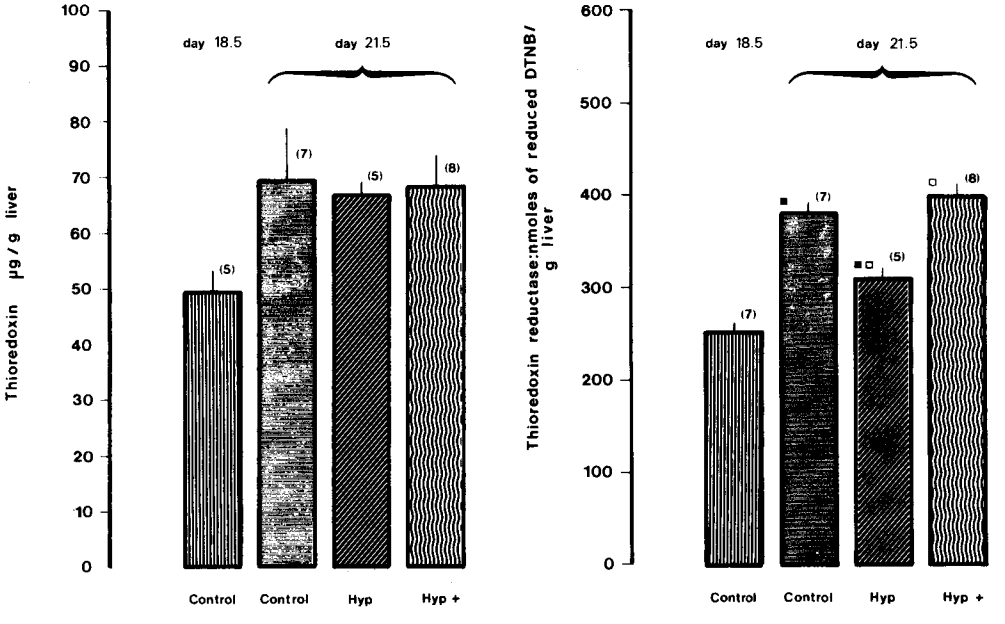

Figure 1. Effect of hypophysectomy on the development of the thioredoxin system. Fetuses from adrenalectomized mothers were hypophysectomized (Hyp) in utero on day 18.5 of pregnancy. Some of them received 50 µg of hydrocortisone acetate (Hyp + cortisol). Thioredoxin level and thioredoxin-reductase activity were measured on day 21.5 of pregnancy and compared with values of 18.5-day-old fetuses. Columns represent the mean ± confidence interval (vertical bars) at p = 0.05. The number of assays is indicated in parentheses.
■ □: Statistically different (p = 0.05).

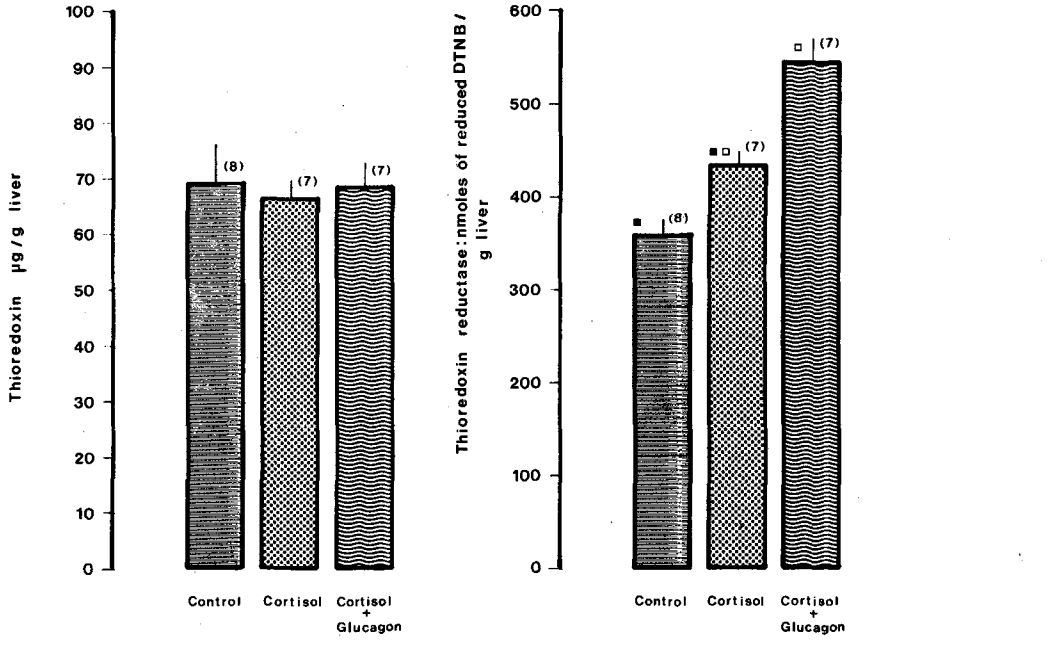

Figure 2. Effect of the administration of 50 µg of hydrocortisone acetate on day 18.5, alone (Cortisol) or in association with 25 µg of glucagon on day 20.5 (Cortisol + Glucagon), on the development of the thioredoxin system. Thioredoxin level and thioredoxin-reductase activity were determined on day 21.5 of pregnancy. Columns represent the mean ± confidence interval (vertical bars) at $p = 0.05$. The number of assays is indicated in parentheses.
■ □: Statistically different ($p = 0.05$).

doxin significantly, increased during the late fetal period, i.e. day 16.5-day 21.5, and showed a marked increase after birth. Thus, if thioredoxin plays a physiological role in neonatal liver, it would be due rather to its reduced state than to its liver concentration. Birth is marked by great changes in metabolic pathways such as hepatic urea synthesis. CPS-I and ASS activities in particular exhibit a marked increase 24 h after birth as a result of the modifications in hormone levels[1,21].

This theory was supported by observations on fetuses treated with corticosteroids and glucagon. Our results showed that the development of thioredoxin-reductase activity requires that the fetal adrenals be intact. The administration of hydrocortisone acetate evoked the enzyme activity prematurely. This stimulatory effect was more marked when glucagon was associated with the corticosteroid hormone. These treatments are without effect on the thioredoxin level. It is concluded that the low level of release of corticosterone by the fetal adrenals during the late fetal period, and the marked increase in both corticosterone and glucagon[18] at birth, might stimulate the activity of thioredoxin reductase. It is known that during this period, glucagon enhances the reduced state of mitochondria[19], stimulates citrullinogenesis[20] and activates CPS-I and ASS in vivo[1,21]. From our previous reports[2,3] it appears that reduced thioredoxin activates CPS-I and ASS in vitro. The rise in thioredoxin-reductase activity at birth might increase the reduced state of thioredoxin. Our hypothesis is that thioredoxin in the reduced state mediates the stimulatory effect of glucagon on CPS-I and ASS activities in vivo. Glucagon might therefore activate CPS-I and ASS by enhancing thioredoxin-reductase activity in vivo. In further studies, we shall investigate the mechanism by which thioredoxin might activate CPS-I and ASS.

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