ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 3, pp. 305-309. © Pleiades Publishing, Ltd., 2008.
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Role of Glucocorticoids and Resident Liver Macrophages in Induction of Tyrosine Aminotransferase

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Received May 14, 2007
Revision received May 25, 2007

Abstract—Administration of cortisol to an animal induces tyrosine aminotransferase (TAT) in the liver. A similar effect was observed after stimulation of resident liver macrophages (Kupffer cells) by dextran sulfate. Actinomycin D completely blocks enzyme induction both by cortisol and dextran sulfate, whereas their combined effect gives an additive result. In primary culture of hepatocytes, dextran sulfate inhibits TAT activity, but conditioned macrophage medium reliably increases enzyme activity in hepatocytes. However, incubation of isolated macrophages in the presence of dextran sulfate and such medium transfer into hepatocyte culture results in even more pronounced increase in TAT activity. In a combined culture of hepatocytes and non-parenchymal liver cells, reproducing intercellular interactions *in vitro*, cortisol and non-parenchymal cells exhibit an additive effect on TAT activity. These results show that liver macrophages release a factor of unknown nature launching the mechanism of TAT induction independently of cortisol, a classic TAT inducer.

DOI: 10.1134/S0006297908030103

Key words: tyrosine aminotransferase, glucocorticoids, macrophage, hepatocyte, liver, intercellular interactions, dextran sulfate

Hydrocortisone (cortisol), cortisone, and corticosterone are assigned to steroid hormones of the adrenal bundle zone (glucocorticoids) and their precursors. Their functions in an organism are diverse and well studied. Among them there are inhibition of glycolysis, enhancement of gluconeogenesis and stimulation of protein synthesis in liver, increased fat mobilization from liver fat reserves stimulated by adrenalin (permissive effect), inhibition of lymphoid system function, anti-inflammatory effect, regulation of cell proliferation, etc. [1, 2]. The effect of glucocorticoids on endocrine organs is connected with lowered production of thyrotropic and adrenocorticotropic hormones and enhanced production of somatotropic hormone. The best studied is the involvement of glucocorticoids in induction of key enzymes of gluconeogenesis such as tyrosine aminotransferase (TAT), tryptophan oxygenase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, etc. [3]. Launching the induction mechanism of these enzymes is associated with interaction of the cytosol receptor of glucocorticoids with the genes responsible for their synthesis. For example, the specific sequence 5′-T-G-T-T-C-T-3′ interacting with glucocorticoid receptor was distinguished in the *TAT* gene structure [3]. The same sequence is present in the long terminal repeat of the mouse mammary tumor virus and in other genes [4].

Investigations on hepatocyte culture have shown that regulation of the *TAT* gene expression is more complicated than described above and is influenced by macrophage secretion products [5]. Thus, the hybrid protein consisting of interleukin-6 and its soluble receptor [6], as well as tumor necrosis factor α [7], cause pronounced dose-dependent inhibition of the *TAT* gene expression in hepatocyte culture. On the contrary, epidermal growth factor (EGF) significantly enhances TAT induction caused by dexamethasone. In this case, EGF itself has no effect on TAT and does not alter TAT induction by glucagon and cAMP [8].

We found previously that liver macrophages (Kupffer cells) are involved in the regulation of protein biosynthe-

Abbreviations: C/EBP) CCAAT transcription factor/enhancer binding protein; DS) dextran sulfate; EGF) epidermal growth factor; Ets) epithelium-specific transcription factor; HDL) high-density lipoproteins; HNF) hepatic nuclear factor; LDL) low-density lipoproteins; NP) non-parenchymal cells; TAT) tyrosine aminotransferase.

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sis in hepatocytes in the intact rats [9] and during liver regeneration [10], and they also alter the metabolism of plasma lipoproteins [11, 12]. This work presents facts indicating that macrophages are involved in induction of TAT in hepatocytes.

MATERIALS AND METHODS

Male Wistar rats of 180-200 g kept on a usual diet were used in this work. To study the effect of liver macrophages on TAT induction in this organ, 500 kD dextran sulfate (Loba Feinchemie, Austria) was used, and the dose (50 mg/kg body weight) was injected into the tail vein [13]. It is known that liver macrophages are involved in elimination of polysaccharide from the organism. It was shown that polysaccharides form in blood complexes with high- and low-density lipoproteins, which are seized by macrophages via "scavenger" receptors, thus changing secretory activity of these cells [14, 15]. The induction of TAT by cortisol was used for comparison. The hormone was injected into rats intraperitoneally 4 h prior to sacrifice at the dose of 5 mg/kg body weight. Actinomycin D (Amersham, England) was used to inhibit DNA-dependent synthesis of RNA. The drug was introduced intraperitoneally at a dose of 1 mg/kg body weight 1 h prior to injection of dextran sulfate (DS) or cortisol. Control rats obtained an equal volume of isotonic solution.

Hepatocytes were isolated using the method of Seglen [16] in our modification [17]. Rats were decapitated under mild ether anesthesia, and a cannula was introduced into the hepatic vein after lancing the abdominal cavity. The liver was perfused for 10 min *in situ* with phosphate buffer without Ca^{2+} and Mg^{2+} to wash the blood off. Then recirculation perfusion with Hanks solution containing 0.03% type I collagenase (Sigma, USA) was performed. After 20-30 min, the liver tissue was mechanically disintegrated using a spatula in a Petri dish. In this case solutions were constantly saturated with carbogene (95% oxygen and 5% CO_2) at pH 7.4 and 37°C. Hepatocytes were separated from non-parenchymal cells and cell debris by centrifugation at 50*g* for 1 min. Non-parenchymal cells were purified from erythrocyte contamination by isopycnic centrifugation in the metrizamide solution [16]. Liver macrophages were obtained from the non-parenchymal cell suspension by counterflow centrifugation at 2500 rpm in a JE-6 elutriator rotor in a J2-21 centrifuge (Beckman, USA) [18].

To reproduce intercellular interactions *in vitro*, the separate and combined cultivation of hepatocytes and non-parenchymal liver cells was used. Freshly isolated hepatocytes were resuspended in the Eagle's MEM medium with 25 mM Hepes, 10% fetal calf serum (Vector, Russia), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). The cells were incubated in 100-mm glass Petri dishes (Anumbra, Italy) covered in advance by type I collagen (Sigma). Final density of hepatocytes was 700 cells/mm^2. To obtain a combined culture of hepatocytes and non-parenchymal liver cells, a monolayer of hepatocytes was prepared first as described above. After 2 h, the medium was removed and Eagle medium containing non-parenchymal cells was added to the hepatocyte monolayer. Two hours later, the combined culture was washed with fresh medium to remove unattached cells and cultivation continued for 24 h. In the case of combined cultivation of hepatocytes and non-parenchymal cells their ratios were 1 : 0.5, 1 : 2, and 1 : 5.

To reveal the role of liver macrophages in TAT activation in hepatocytes, freshly isolated cells were incubated at 37°C in Eagle's medium containing the abovementioned components. To stimulate the macrophage secretory activity, DS was added to the medium (25 μg/ml). After 4.5 h the conditioned medium was separated from cells by centrifugation at 300*g* for 10 min, sterilized using a filter with pore diameter of 0.45 μm (Millipore, USA), and transferred to the 24-h culture of hepatocytes. The incubation of hepatocytes continued for 4 h. Then the cellular monolayer was washed with cooled isotonic solution and cells were removed using a silicon rubber.

The TAT activity was determined by the formation of *p*-hydroxybenzaldehyde from *p*-hydroxyphenylpyruvate in alkaline medium at the wavelength of 331 nm [19]. Reaction medium consisted of 6 mM sodium phosphate buffer, pH 7.3, 15 mM L-tyrosine (Sigma), 0.12 μM pyridoxal 5-phosphate, 12 μM sodium diethyldithiocarbamate, and 40 μg (by protein) of the tissue or cell supernatant (after centrifugation at 50,000*g* for 30 min). The reaction was initiated by addition of 0.3 mM α-ketoglutarate and stopped by addition of 10 M NaOH. Measurements were carried out 30 min later using a Hitachi spectrophotometer (Japan). The enzyme activity was expressed in nanomoles of *p*-hydroxyphenylpyruvate/min per mg protein. Statistical data processing was carried out using the ANOVA test and the Microcal Origin program (USA).

RESULTS AND DISCUSSION

Stimulation of resident liver macrophages by DS in *in vivo* experiments resulted in a sharp increase in TAT activity in this organ. Already after 1 h, TAT activity was twice as high as in non-stimulated animals (Fig. 1). Maximal increase in the enzyme activity (sevenfold) was observed 3 h after DS injection, and then it decreased gradually but did not achieve the initial level up to the end of the observation (24 h). Thus, the macrophage stimulation by polysaccharide sulfates results not only in pronounced but in long-term increase in the enzyme activity in liver, i.e. de-induction is a slow process.

To compare the effect of DS with that of a classic TAT inducer, cortisol, we evaluated the enzyme activity

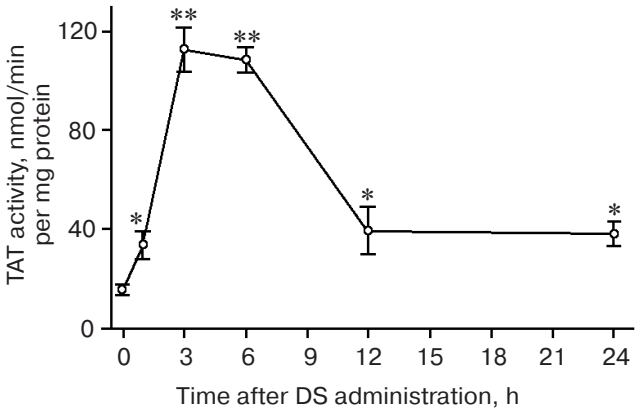

Fig. 1. Dynamics of TAT activity changes in rat liver at different times after intravenous administration of DS at a dose of 50 mg/kg. Reliable changes relative to control (0 h): * $p < 0.05$, ** $p < 0.001$.

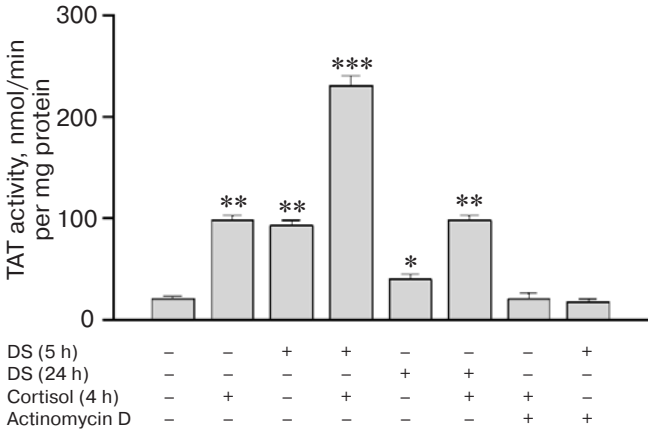

Fig. 2. TAT induction in rat liver upon separate and combined action of cortisol and DS. Cortisol (5 mg/kg) was injected intraperitoneally 4 h prior to taking the material, DS (50 mg/kg) 1 h or 24 h prior to cortisol injection, actinomycin D (1 mg/kg) was injected intraperitoneally 1 h prior to cortisol and DS injection. Reliable changes relative to control (without additions): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

upon DS intravenous administration, intraperitoneal injection of cortisol, and after administration of both preparations. It was shown that in the case of DS administration, after 5 h the TAT activity in liver increased 4.6-fold in comparison with control (Fig. 2). Cortisol injection resulted in 4.8-fold increase in enzyme activity after 4 h. After combined administration of DS and the hormone the enzyme activity increased 10.8-fold ($p < 0.01$). The detected additive effect of cortisol and DS on TAT activity is indicative of the existence of two independent mechanisms of TAT induction. The effect of hydrocortisone is realized via the known cytosol receptor of the hormone, whereas the DS effect is realized via macrophage stimulation and secretion of factors enhancing expression of corresponding genes. Both mechanisms are connected with the mechanism of enzyme induction because transcription inhibition by actinomycin D 1 h prior the DS or cortisol administration completely inhibited TAT induction. It should be noted that there was no additive effect in the case of the hormone administration to animals 24 h after macrophage stimulation by DS, though the initial TAT activity in this group of animals was double compared with the control. Evidently, this can be explained both by significant decrease at this time of production by macrophages of factors involved in TAT induction and by the effect of a de-induction mechanism.

Results obtained in experiments on animals do not allow unambiguous detection of the role of macrophages in TAT regulation because under these conditions one cannot exclude both the direct effect of preparations on hepatocytes or their mediated effect via macrophages of other sections of the mononuclear phagocyte systems. Owing to this, we investigated cultures of hepatocytes incubated in conditioned medium of DS-stimulated liver macrophages. It appeared that DS addition to the hepatocyte incubation medium reliably decreased the TAT activity (Table 1). On the contrary, the conditioned medium of macrophages reliably increased the enzyme activity. Incubation of macrophages in the presence of DS and

Table 1. Effect of conditioned medium of Kupffer cells incubated for 4.5 h in the presence of DS on the TAT activity in the hepatocyte culture

Cultivation conditions	TAT activity, nmol/min per mg protein
MEM medium (control)	4.2 ± 0.2
MEM medium + DS	3.1 ± 0.2*
Conditioned medium of Kupffer cells	4.9 ± 0.3*
Conditioned medium of Kupffer cells + DS	6.7 ± 0.5**

Note: Prior to the beginning of the experiment, hepatocytes were cultivated for 24 h in MEM medium containing 25 mM Hepes and 10% fetal calf serum. To obtain conditioned medium, Kupffer cells were incubated for 4.5 h in a similar MEM medium in the presence of DS (25 μg/ml). TAT activity in hepatocytes was determined after their incubation for 4 h in conditioned medium of Kupffer cells. Hepatocytes incubated for 4 h in MEM or in MEM containing DS (25 μg/ml) were used as control [13]. Results are shown as $M \pm m$ of three experiments, two Petri dishes being used in each experiment.

* Reliable changes ($p < 0.05$) compared to control (culture of hepatocytes incubated in MEM).

** Reliable changes ($p < 0.05$) compared to the culture of hepatocytes incubated in conditioned medium of Kupffer cells.

Table 2. TAT induction by cortisol in hepatocyte culture and in combined culture of hepatocytes with non-parenchymal (NP) liver cells

Experimental conditions	TAT activity, nmol/min per mg protein			
	hepatocyte culture	hepatocyte/NP cell ratio in combined culture		
		1 : 5	1 : 2	1 : 0.5
Without hormone	2.1 ± 0.4	5.6 ± 0.7*	5.0 ± 0.2*	2.5 ± 0.4
Cortisol	10.5 ± 1.5	16.0 ± 1.8*#	17.9 ± 2.1*#	15.3 ± 1.5*#

Note: Hepatocytes were cultivated separately or in a combined culture with NP cells for 24 h in MEM containing 25 mM Hepes and 10% fetal calf serum. Cortisol (10 μmol) was added 4 h prior to the end of cultivation.

* Reliable changes ($p < 0.05$) in combined culture compared with the hepatocyte culture incubated with cortisol or without hormone.

\# Reliable changes ($p < 0.01$) compared with combined culture incubated without hormone.

such medium transfer to the hepatocyte culture resulted in even more pronounced increase in TAT activity (Table 1). These results show that macrophages produce a factor of unknown nature, which enhances enzyme induction in hepatocytes. The DS-activated macrophages produce larger amounts of this factor.

The independence of mechanisms of TAT induction in hepatocytes, influenced by macrophages and cortisol, can be also traced upon separate and combined cultivation of hepatocytes with non-parenchymal cells enriched with liver macrophages. It is known that the cortisol-dependent TAT induction in liver takes place only in hepatocytes [20]. No TAT activity was revealed in suspension of non-parenchymal cells used for preparation of combined culture. Table 2 shows that addition of cortisol to the primary culture of hepatocytes increased TAT activity five-fold. Combined incubation of hepatocytes with non-parenchymal cells at a ratio of 1 : 5 increased enzyme activity 2.6-fold. Lowering the amount of non-parenchymal cells in combined culture to the ratio of 1 : 2 had already a less pronounced inducing effect. After decreasing this ratio to 1: 0.5, the stimulating effect was practically absent. Introduction of cortisol into combined culture of these cells at a ratio of 1 : 5 resulted in an additive effect, and at ratios of 1 : 2 and 1 : 0.5 hormone even somewhat enhanced this effect. Thus, hormone increased TAT activity three-fold in the combined culture with the ratio of 1 : 5 and six-fold at the ratio of 1 : 0.5 (Table 2).

So the mechanism of TAT induction in liver is more complex than it seemed previously. The best studied is the effect of glucocorticoids. It was shown that in this case the induction of the *TAT* gene involves the cooperative interaction of two glucocorticoid-sensitive elements localized in the region of –2500 and –5500 bp [21]. The glucocorticoid-sensitive TAT element consists of numerous adjacent and overlapped binding sites for glucocorticoid receptor and other transcription factors including those of C/EBP, HNF-3, and Ets families [22-24]. The discovery of the macrophage-dependent mechanism of TAT induction allows us to suppose that the promoter region of this gene contains regulatory sites involved in paracrine regulation of macrophage expression. However, it is not clear how the additive effect of two different mechanisms of regulation of the gene expression is achieved. Perhaps there are two copies of the gene of the enzyme in the hepatocyte genome. One is regulated by glucocorticoids. The other is under control of a factor produced in macrophages. Activation of the latter by polysaccharides enhances production of this factor and launches an additional induction mechanism. If both mechanisms are launched simultaneously, the additive result of their action is observed.

The role of liver macrophages in paracrine regulation was also demonstrated for other proteins of hepatocytes. The best studied is induction of proteins of the acute phase of inflammation. It has been shown that incubation of liver macrophages with lipopolysaccharides causes secretion of factors with molecular masses of 30 and 100 kD, stimulating synthesis of α-2-macroglobulin in hepatocytes [25]. However, unlike the effect detected by us for TAT, induction of α-2-macroglobulin is observed only in the presence of dexamethasone, i.e., the synergism in action of glucocorticoids and the macrophage secretion products is required [25, 26]. The nature of the macrophage factor involved in TAT induction is still not clear.

Authors are indebted to A. V. Kharkovski for technical assistance in experiments on the liver cell culture.

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