The influence of corticosterone on antizyme gene expression in early regenerating rat liver

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Abstract

Background and study aims : **Antizyme 1, a specific inhibitor of Ornithine decarboxylase (ODC), plays a critical role in cell proliferation. Little is known about the impact of glucocorticoid on antizyme expression in the regenerating liver. In this paper, the effect of corticosterone on the gene expression of antizyme 1 in early regenerating rat liver induced by partial hepatectomy (PH) was investigated.**

Materials and methods : **Bilateral adrenalectomies (ADX) were performed 3 days before PH. Corticosterone in sesame oil or sesame oil was injected sub-cutaneously to ADX rats. Antizyme 1 mRNA and protein levels as well as polyamine contents in the regenerating liver were determined by RT-PCR, Western blotting and HPLC, respectively.**

Results : **Antizyme 1 protein content in the oil-treated ADX group decreased significantly at 5, 7 and 9 h after PH compared to control. Following corticosterone administration the content rose dose-dependently during the whole experiment. At 5 h post-PH, the protein levels in 10 and 40 mg/kg corticosterone-treated ADX rats increased by 66% and 148%, respectively, when compared with the control group. However, no significant changes in antizyme 1 mRNA levels were observed in oil-treated ADX rats or corticosterone-treated groups compared to control. Polyamine contents in oil-treated ADX rats were the highest among all groups at 5 and 9 h. Corticosterone treatment resulted in a dramatic decrease of polyamine contents at most of the time points investigated when compared with those in control rats.**

Conclusions : **Corticosterone treatment induces antizyme 1 protein synthesis in early regenerating rat liver. However, it has little effect on antizyme 1 gene transcription.** (Acta gastroenterol. belg., **2011**, 74, **289-294**).

Key words : corticosterone, antizyme, gene expression, regenerating liver, rat.

Introduction

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the biosynthesis of polyamines (putrescine, spermidine, spermine) essential for growth and differentiation of mammalian cells (1). ODC activity has been demonstrated to be intimately associated with liver regeneration. After partial hepatectomy (PH), hepatocytes rapidly enter the cell cycle and the remnant liver undergoes a process of proliferation, during which both ODC expression and its activity dramatically increase before DNA synthesis. Therefore, ODC is considered to initiate rat liver regeneration (2-4). Glucocorticoid is one of the most important hormones in regulating liver regeneration. Our previous work has shown that corticosterone (the main glucocorticoid in rat) treatment promotes the proliferation of hepatocytes (5), and further studies indicate that the hormone causes a decrease in ODC activity which partially correlates with the changes in both ODC mRNA and protein levels in the regenerating liver (6).

Regulation of ODC activity at the post-translational level has also been demonstrated (7). Post-translational regulation of ODC is mainly attributed to the actions of antizymes, a family of at least three proteins including antizyme 1, antizyme 2 and antizyme 3 (8). Antizyme 1 is by far the most studied member, which binds to the ODC subunit to form enzymatically inactive heterodimers. The affinity of antizyme 1 for ODC subunit is higher than that between ODC subunits themselves. Interaction between antizyme 1 and ODC subunits has two outcomes : First, ODC is inactivated ; second, the ODC subunits are targeted for degradation by the 26S proteasome without ubiquitination (9-11). The relationship between antizyme 1 expression and tumor cell proliferation has been well studied (12-14). However, little is known about the *in vivo* expression of antizyme in regenerating hepatocytes, and, in particular, the influence of glucocorticoid on the enzyme. In this paper, the regenerating rat liver was used to analyze the regulatory effect of corticosterone on antizyme 1 gene expression.

Materials and Methods

Animals and treatment

The animal experiments were conducted in compliance with the Declaration of Helsinki guidelines for the care and use of animals in research.

Adult male Sprague–Dawley rats (180 to 200 g) from Henan Normal University were housed in an air-conditioned room maintained at 23 ± 2°C on a 12 h light–dark cycle. They had free access to food and tap water ad libitum. The rats were randomly subdivided into eight groups : a sham-adrenalectomy (sham-ADX) group, a sham-ADX group injected with sesame oil (control), a ADX group, a ADX group injected with oil, a ADX group treated with 10 or 40 mg/kg body weight

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corticosterone, and a sham-ADX group treated with 10 or 40 mg/kg body weight corticosterone. Both ADX and PH operations were performed as described (6). Corticosterone in sesame oil or sesame oil was injected subcutanously to ADX or sham-ADX rats 12 h before PH and at the time of PH, ADX rats were given 0.9% NaCl in the drinking water. Because the rats were injected twice.

Rats were sacrificed at 0, 3, 5, 7, 9 and 12 h after PH. The right lobe of the rat livers was rapidly removed and frozen in liquid nitrogen, and then maintained at -80°C. Twelve to eighteen animals were included in each group.

Reagents

Reagents were purchased as follows : corticosterone from N.V. Organon (OSS, the Netherlands) ; Trizol Reagent from Invitrogen (Carlsbad, California, USA) ; M-MLV one step RT-PCR Kit from Sangon (Shanghai, China) ; DIG Nucleic Acid Detection Kit from Roche (Mannhein, Germany) ; polyclonal rabbit anti-rat antizyme 1 (Matsufuji S *et al.* 1990. J Biochem (Tokyo) 108 :365-371) ; β-actin mouse monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) ; alkaline phosphatase conjugated anti-rabbit IgG or anti-mouse IgG from Zhongshan Jinqiao Biotechnology (Beijing, China) ; pyridoxal-5-phosphate, putrescine, spermidine and spermine from Sigma (St Louis, USA).

RNA isolation and RT-PCR

Total RNA from the liver tissue was extracted using the Trizol reagent according to the manufacturer's protocol. RT and PCR reactions were performed with the M-MLV one step RT-PCR kit. The housekeeping gene GAPDH served as an internal standard. The parameters were as follows : 30 min at 40°C for the RT reaction, 30 s at 94°C, 30 s at 57°C, 30 s at 72°C for 23 cycles (antizyme) or for 21 cycles (GAPDH). Primers were as follows : antizyme 1 (NM139081) forward-GAA GGC AGC AAG GAC AGT and reverse-CCC CGC TAA TGG AGT GAG, GAPDH (NM017008) forward-GGC AAG TTC AAG GCA CAG and reverse-CGC CAG TAG ACT CCA CGA CA.

Two µl of the reaction products were separated by electrophoresis in the presence of ethidium bromide to confirm the predicted size under an ultraviolet transilluminator before transferred to nylon membranes, and then hybridized with the digoxin-endlabeled oligonucleotide probes (antizyme GGA GTA GGG CGG CTC TGT CCT CAC GGT TCT, GAPDH GGT CAT CAA CGG GAA ACC CAT CAC CAT CTT). Subsequent detection of the bound probes was performed using a DIG Nucleic Acid Detection Kit. The results were scanned and the optical density of each band was measured using the ChampGEL (Sage Creation, Beijing, China).

Antizyme protein analysis

Liver tissue was homogenized at 0°C in 0.1 mol/L TBS containing 1 mmol/L NaF, 1 mmol/L Na_3O_4V, 5 mmol/L EDTA, and 1 mmol/L PMSF, then centrifuged at 10 000 × *g* for 20 min at 0°C. Protein concentrations in the supernatant were determined using the Bradford protein assay. β-actin served as an internal standard. One hundred (100) µg of samples were subjected to 15% PAGE containing SDS and transferred to nitrocellulose membranes at 150 mA for 90 min. Immunoblots were washed twice in 0.1 mol/L TBS + 0.1% Tween 20 (TBST) and immersed in 3% BSA for 1 h at 37°C. The blots were incubated with antizyme 1 (1:2000 dilution) or β-actin primary antibodies (1:1000 dilution). Membranes were washed four times for 15 min, and the blots were then incubated with either alkaline phosphatase conjugated anti-rabbit IgG (1:3000 dilution) or anti-mouse IgG (1:2000 dilution), followed by a second extensive wash.

Measurement of polyamine contents

Four hundred mg of liver was washed in ice-cold PBS, homogenized in 150 mmol/L PBS containing 1.5 mmol/L EDTA, 2.5 mmol/L dithiothreitol and 75 nmol/L pyridoxal-5-phosphate and centrifuged at 10000 × g for 5 min at 0°C. The supernatant was collected and stored at -20°C for analysis.

Polyamine levels were determined by HPLC as previously described (15). The chromatogram conditions were as follows : Attach C_{18} (150 × 4.6, 3 µm), methanol : double distilled water (65 :35), 0.8 mL/min ; wave length : 232 nm.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Significant differences between means were determined by a one-way ANOVA followed by the Dunnett's test. All statistical analyses were performed using SPSS version 11.5.

Results

Effect of corticosterone on antizyme 1 mRNA levels in the regenerating liver

In the control group, antizyme 1 mRNA expression was relatively low in intact liver. After PH, levels increased and peaked at 3 h, followed by a decrease at 5 h, another peak at 7 h, and then declined gradually (Fig. 1A, E).

In both 10 and 40 mg/kg corticosterone-treated ADX groups as well as oil-treated ADX group, antizyme 1 mRNA levels changed slightly during the experiment but without reaching statistical significance compared to control (Fig. 1B-E).

The results indicate that corticosterone treatment has little effect on antizyme 1 mRNA transcripts in early regenerating rat liver.

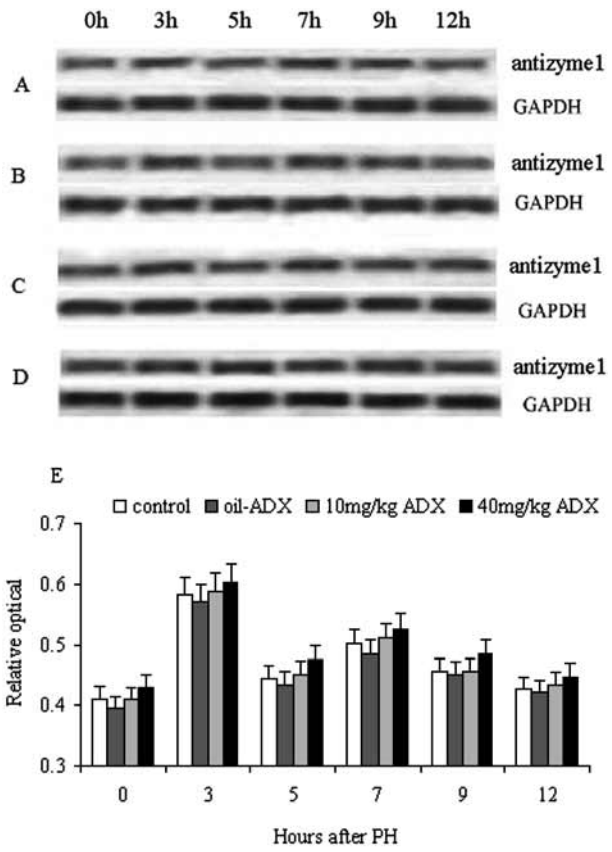


Fig. 1. — Effect of corticosterone on antizyme 1 mRNA expression in rat regenerating liver.
A-D : Southern blotting of RT-PCR products. A : control ; B : adrenalectomy (ADX) + sesame oil (oil ADX) ; C : ADX +10 mg/kg body weight corticosterone (10 mg/kg ADX) ; D : ADX + 40 mg/kg body weight corticosterone (40 mg/kg ADX) ; E : Time-course of antizyme 1 mRNA levels during early liver regeneration. One representative experiment is shown, n = 3/each time point/each group (the same below).

Stimulatory effect of corticosteron on antizyme 1 protein levels in regenerating liver

The amount of antizyme 1 protein was measured using Western blotting. Two bands of 25 kDa and 29 kDa (Fig. 2A-F), respectively, which represented different forms of antizyme protein were observed. Only the 25 kDa band was analyzed in this paper because the other (29 kDa) one was too faint to be quantified accurately.

In the control group, expression profile of 25 kDa protein resembled that of antizyme 1 mRNA, with two peaks at 3 and 7 h post-PH, respectively (Fig. 2A, G). The contents of the protein in the oil-treated ADX group were similar to that in controls at 0, 3 and 12 h post-PH, but declined significantly by 17.5%, 12.5% and 18% at 5, 7 and 9 h post-PH, respectively (Fig. 2B, G).

Corticosterone administration resulted in an alteration of the protein expression pattern in ADX rats. The contents rose significantly not only in intact liver but also in the regenerating one during the whole experiment, with the highest value occurring at 5 h post-PH. The levels in the10 and 40 mg/kg ADX groups increased by 66% and 148%, respectively, when compared with control (Fig. 2C, D, G). Additionally, the protein levels observed in the 10 and 40 mg/kg corticosterone-treated ADX rats were lower than those in sham-ADX rats treated with corticosterone at the corresponding doses. For example, a 15.1% and 17.3% decrease were detected at 7 h post-PH, respectively (Fig. 2E, F, H), further demonstrating that both exogenous and endogenous corticosterone stimulated antizyme 1 protein expression in the regenerating rat liver.

In an additional experiment, we investigated the effect of sesame oil injection on the expression of antizyme 1 and polyamine levels showing no statistical change (data not shown).

Effect of corticosterone on polyamine contents in the regenerating liver

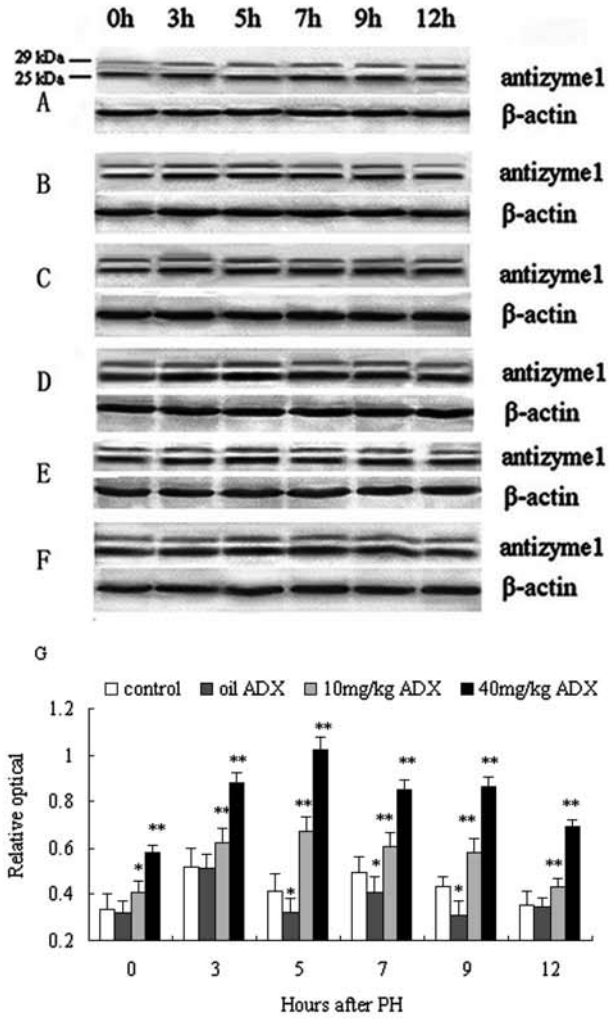
During the early period of liver regeneration , the trends in the change of the 3 polyamine contents were similar in all groups, very low in intact liver, but increasing after the operation with a peak value at 5 h, followed by a gradual decrease thereafter (Table 1).

Polyamine contents detected in oil-treated ADX rats were the highest among all groups. Following corticosterone treatment, however, the levels declined dramatically, especially at 5, 9 and 12 h post-PH compared to the control group, but remained still higher than those in sham-ADX group with corresponding dose of corticosterone treatment at 5 or 9 h (Table 1), showing an inhibitory effect of corticosterone on polyamine levels.

Discussion

A surgical operation such as PH, an intense stressor to rat, can cause an increase in glucocorticoid concentration (16). Generally, glucocorticoid acts through mediating gene transcription, but in this experiment, no significant difference in antizyme mRNA levels is observed after 10 or 40 mg/kg of hormonal treatment by means of semi-quantitative PCR, suggesting that the effect of glucocorticoid on antizyme transcription is not conspicuous. Similarly, it has been reported that dexamethasone has no regulatory effect on antizyme mRNA expression in postnatal rat liver (17). It is likely that there is no glucocorticoid response element in the region of the antizyme gene or that glucocorticoid could not bind to the antizyme transcription factor. However, it is necessary to further confirm what we observed using more precise real time quantitative PCR.

It is unique for antizyme transcription because there are two alternative translation start sites in antizyme mRNA (10,18). As a result, two different forms of antizyme protein are expressed. The 25 kDa protein is



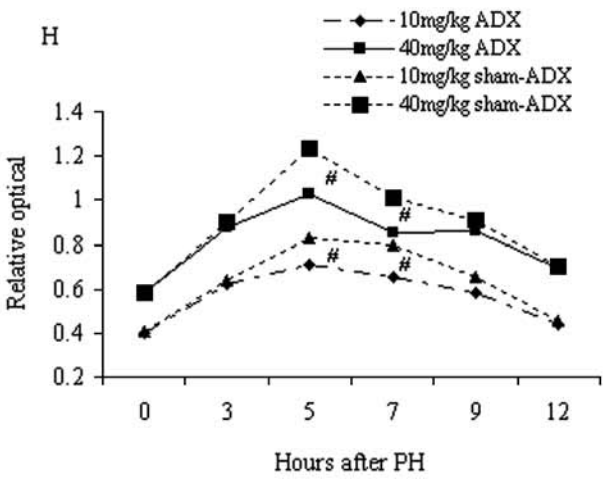


Fig. 2. — Stimulation of antizyme 1 protein expression by corticosterone during liver regeneration. A-F : Western blotting of antizyme 1 protein. A : control ; B : adrenalectomy (ADX) + sesame oil (oil ADX) ; C : ADX +10 mg/kg body weight corticosterone (10 mg/kg ADX) ; D : ADX + 40 mg/kg body weight corticosterone (40 mg/kg ADX) ; E : sham-ADX + 10 mg/kg body weight corticosterone (10 mg/kg sham-ADX) ; F : sham-ADX + 40 mg/kg body weight corticosterone (40 mg/kg sham-ADX) ; G- H : Time-course of antizyme 1 protein expression during early liver regeneration. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to control group at the corresponding time point ; $^{\#}P < 0.05$, compared to sham-ADX group treated by 10 or 40 mg/kg body weight corticosterone at the corresponding time point.

more abundant due to the more efficient utilization of the second start site (19). Our results show that antizyme protein is vastly stimulated in a dose-dependent manner following corticosterone treatment during the whole experiment which is different from its mRNA expression. Endogenous glucocorticoid also promotes antizyme 1 protein expression, as the protein content in oil-treated ADX animals decreases statistically during 5-9 h post-PH. This time-course-delayed effect of the hormone on antizyme 1 may be attributed to the decreasing level of intrahepatic glucocorticoid as a result of the stress in response to PH. The accumulation of corticosterone-induced antizyme 1 protein reaches the maximum at 5 h post-PH, which is consistent with our previous data that both ODC protein and activity fall significantly under the same conditions, with the lowest values occurring at 5 and 6 h post-PH, respectively (6). It is well known that an increase in antizyme accelerates ODC degradation, which leads to a loss of ODC protein and activity (9,11).

It is widely accepted that antizyme is synthesized in response to increased cellular polyamine contents through a unique ribosomal frame-shift mechanism (10). The increment of antizyme promotes the degradation of ODC, thus restricting polyamine biosynthesis. Considering the close relationship between ODC, antizyme and polyamines, endogenous putrescine, spermidine and spermine levels were determined for all the groups at some main time points. Our data reveal that the polyamine levels were greatly reduced after corticosterone administration, which is most likely due to the decline in ODC activity (6). We presume that antizyme 1 may be directly up-regulated by corticosterone, which is one of the mechanisms by which the hormone inhibits ODC during early rat liver regeneration. Furthermore, over expression of antizyme 1 not only inhibits polyamine up-take but also stimulates polyamine excretion (20,21), while the reduction of polyamine is reported to be responsible for an impairment to initiate liver regeneration (22). Maybe this is one of the molecular mechanisms by which corticosterone inhibits liver regeneration (23).

In conclusion, our results indicate that exogenous corticosterone dose-dependently stimulates antizyme 1 expression. This regulatory effect mainly occurs at the post- transcriptional level. Nevertheless, the influence of the hormone on actual antizyme activity needs to be further investigated.

Table 1. — **Effect of corticosterone on polyamine contents of the regenerating live**

Polyamines	Group	Hours after PH				
		0 h	3 h	5 h	9 h	12 h
Putrescine (nmol/mg liver)	A	2.11 ± 0.44	2.93 ± 0.73	8.81 ± 0.74	7.66 ± 0.50	7.02 ± 0.59
	B	2.88 ± 0.44	3.51 ± 0.62	12.55 ± 0.95**	9.33 ± 0.50*	8.15 ± 1.64
	C	1.46 ± 0.63	1.98 ± 0.57	7.1 0± 0.89**#	5.31 ± 0.90*	4.17 ± 0.93*
	D	0.97 ± 0.41*	1.49 ± 0.53**	5.73 ± 0.73**#	3.50 ± 0.34**#	1.46 ± 0.65**
	E	1.40 ± 0.49	1.79 ± 0.53	6.01 ± 0.80	4.71 ± 1.10	3.97 ± 0.65
	F	0.89 ± 0.39	1.32 ± 0.53	4.79 ± 0.87	2.60 ± 0.64	2.16 ± 0.59
Spermidine (nmol/mg liver)	A	5.06 ± 1.04	6.71 ± 0.94	19.91 ± 1.18	17.07 ± 0.94	15.98 ± 1.13
	B	6.11 ± 1.05	7.16 ± 1.19	26.41 ± 1.40*	20.96 ± 1.21**	16.74 ± 1.60
	C	3.56 ± 0.71	4.97 ± 0.88*	17.33 ± 1.47*#	12.7 ± 1.45*	9.61 ± 1.03**
	D	2.17 ± 0.71**	3.31 ± 0.70**	12.03 ± 1.08**#	7.36 ± 1.12**	5.28 ± 1.04**
	E	3.26 ± 0.59	4.24 ± 0.67	13.12 ± 0.81	10.28 ± 1.10	8.96 ± 0.86
	F	2.01 ± 0.67	2.98 ± 0.71	9.79 ± 1.23	6.50 ± 0.66	4.98 ± 0.98
Spermine (nmol/mg liver)	A	7.65 ± 0.70	10.21 ± 1.65	29.21 ± 0.95	35.03 ± 1.85	22.44 ± 0.62
	B	9.21 ± 1.21	12.32 ± 1.14	41.59 ± 1.40**	30.32 ± 1.21**	24.32 ± 1.60
	C	6.57 ± 0.99	7.61 ± 0.90*	23.51 ± 1.61*#	17.59 ± 0.74*	13.83 ± 1.20**
	D	3.67 ± 0.79**	5.45 ± 0.65**	19.05 ± 1.39**##	10.52 ± 1.49**#	7.64 ± 1.13**
	E	4.89 ± 0.58	6.08 ± 0.99	20.07 ± 1.21	14.32 ± 1.10	12.97 ± 1.11
	F	3.24 ± 0.76	5.03 ± 0.65	15.46 ± 0.67	8.53 ± 0.78	6.68 ± 1.05

A : control ; B : adrenalectomy (ADX) + sesame oil ; C : ADX +10 mg/kg body weight corticosterone ; D : ADX + 40 mg/kg body weight corticosterone ; E : sham-ADX + 10 mg/kg body weight corticosterone ; F : sham-ADX + 40 mg/kg body weight corticosterone. *$P < 0.05$, **$P < 0.01$, compared to control group at the corresponding time point ; #$P < 0.05$; # #$P < 0.01$, compared to sham-ADX group treated by 10 or 40 mg/kg body weight corticosterone at the corresponding time point.

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