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In vivo quantification of removal of asialo-orosomucoid from the circulation in anaesthetized streptozotocin-diabetic rats

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Summary. The in vivo kinetic of removal of ^{3}H asialo-orosomucoid from plasma was investigated in control and streptozotocin-diabetic rats after intravenous injection of 1 mg of asialo-orosomucoid/100 g body wt. Michaelis-Menten kinetics of disappearance were observed. In diabetic rats the maximal rate (Vmax) of disappearance of ^{3}H asialo-orosomucoid was decreased by 30% with no modification of Michaelis constant. Since no accumulation of desialylated orosomucoid in the circulation was observed, the slower rate of removal of ^{3}H asialo-orosomucoid was attributed to a decrease in the number of hepatic asialoglycoprotein receptors which are largely involved in the catabolism of asialoglycoproteins. Our estimate on in vivo maximal rates was 10- to 20-fold greater than our previous in vitro estimate of the maximal rate of endocytosis. In contrast, the values of the Michaelis constant obtained in vivo and in vitro were very similar.

Key words: Asialo-orosomucoid, streptozotocin-diabetes, glycoprotein.

A specific receptor which binds and mediates the endocytosis of desialylated glycoproteins is present at the outer surface of hepatocytes [1, 2]. This receptor-mediated endocytosis is a general phenomenon which concerns all the glycoproteins, provided the sialic acid residues are removed; therefore the galactose residues are at the ultimate position. However, the efficiency of removal from the circulation depends on the structure of the glycan moiety. Asialo-orosomucoid (ASOR), which contains a high percentage of carbohydrates (40%), is most rapidly taken up by liver. Furthermore, the asialoglycoproteins receptor expression is affected by the state of an animal and particularly by the growth state of cells [2].

We have recently reported that the binding and uptake of ^{3}H ASOR by hepatocytes from streptozotocin-diabetic rats were dramatically altered when compared to their removal by normal rat hepatocytes. This was related to a decrease in the number of cell surface receptors with no modification in the apparent affinity constants [3]. The number of intracellular receptors was also decreased [4]. Since the reported number of cell surface receptors is highly variable, depending on experimental conditions used in regard to liver cell isolation and incubation [5], it seems difficult to predict the in vivo behaviour of injected ^{3}H ASOR from our in vitro results [3]. To this purpose, ^{3}H ASOR was injected intravenously in normal and diabetic rats, and its disappearance from plasma was measured. The presence of partially desialylated orosomucoid in plasma was also investigated.

Materials and methods

Experimental animals

Sprague-Dawley rats (male, 51-55 days old) were used for all experiments. Diabetes was induced by a single intravenous injection (dorsal tail vein) of streptozotocin (65 mg/kg body wt.) dissolved immediately before use in 0.2 mol/l citrate buffer isotonic, pH 4.6. Control animals were injected with buffer alone. Fourteen to 20 days after streptozotocin injection, diabetes was determined by a 24% weight loss, plasma-glucose levels between 5.80 g/l to 6.79 g/l and glycosuria. No protein and no ketone bodies were detected in urine using albustix (sensitivity 50 mg/l) and ketostix (sensitivity 50 mg/l) respectively (Ames, Paris, France). Hematocrit was 0.47 ± 0.03 and natraemia was 140 ± 2 mmol/l for control and diabetic rats respectively. Hence the diabetic rats were free of severe acute or chronic complications such as nephropathy and dehydration. The rats had free access to food until the morning of the experiment.

Reagents

Human orosomucoid was a gift from Dr. Wickerhauser (American Red Cross, NIH, Bethesda, MD, USA). Agarose-immobilized neuraminidase type X.A and streptozotocin were purchased from Sigma

Chemical Co. (St Louis, MO, USA), tritiated sodium borohydride (specific activity 60 Ci/mmol) from Commissariat de l'Energie Atomique (Gif-sur-yvette, France), Dynagel from Comptoir Lyonnais de Verreries Interbio (Lyon, France) and Sephadex G 25 from Pharmacia (Uppsala, Sweden). Sprague-Dawley rats were purchased from Charles River (St Aubin Les Elbeuf, France). All other reagents were laboratory grade and obtained from local sources.

Determination of serum orosomucoid and its desialylation by immunological methods

Before the injection of ^{3}H ASOR, a blood sample was drawn by the jugular vein and serum orosomucoid was measured by two immunological methods: the radial immunodiffusion method (RID) of Mancini [6] and the electroimmunodiffusion method (EID) of Laurell [7] as described by Biou et al. [8]. The percentage of the underevaluation by electroimmunodiffusion relative to radial immunodiffusion was calculated. Then, using previously established standard curves of the relation between the percentage of desialylation and the percentage of underevaluation, the degree of desialylation of orosomucoid was calculated [8].

Desialylation and radiolabelling of orosomucoid

Human orosomucoid was desialylated with agarose-immobilized neuraminidase. The reductive methylation of Wilder [9] was used to generate ^{3}H ASOR.

Typically 10 mg of protein in 1 ml of 0.2 mol/l sodium borate buffer pH 9 were mixed on ice with 18 µl of 3.5% aqueous formaldehyde and added to a freshly opened ampoule containing 25 mCi of tritiated sodium borohydride. After 30 min, the labelled protein was purified by gel filtration on Sephadex G25, the eluting buffer being 0.005 mol/l $NaHCO_3$/0.15 mol/l NaCl, pH 7. ^{3}H ASOR can be stored at -20 °C for months with no loss of activity and no degradation. The specific activity was 4 Ci/mmol.

Catheterization and blood sampling

The animals were anaesthetized with sodium pentobarbital (50 mg/kg body wt.). Jugular vein and penial vein were then exposed and cannulated with silastic Dow Corning tubing and PE 50 tubing filled with heparinized saline respectively. A pretest blood sample was taken for the determination of hematocrit and plasma glucose. Doses of 1000 µg of ASOR per 100 g of body weight were injected. At time 0, the ^{3}H ASOR at a specific radioactivity of 4 Ci/mmol, mixed with unlabelled asialoglycoprotein in a total volume of about 0.5 ml 0.9% NaCl, was rapidly injected via the penial catheter. The catheter was flushed through one time with 0.5 ml of saline and heparin to remove any trace of radioactivity. Blood samples (200 µl) were taken at sampling times of 1, 2, 3, 4, 5, 8, 10, 15, 20, 30, 45 and 60 min. Blood was aspirated via the jugular catheter first to dispel the heparinized saline and the sample was then drawn into a clean syringe. On the last blood sample, hematocrit was measured. There was no significant haematocrit difference between control and streptozotocin-treated rats, nor between the first and the last samples of the experiment. The amount of radioactivity detected in the urine formed during the experiment represented 1p100 of the dose injected in control as well as diabetic rats.

Determination of ^{3}H asialo-orosomucoid in plasma

Each blood sample was transferred to a microfuge tube, and plasma was separated by centrifugation. All specimens were assayed in duplicates. Total radioactivity was determined in a Kontron liquid scintillation counter (Betamatic Intertechnique, Trappes, France) using 30 µl of plasma and 10 ml of Dynagel (Comptoir Lyonnais de Verreries, Interbio, Lyon, France) as the scintillant. Deproteinization was carried out with 40 µl of 10% (w/v) phosphotungstic acid in 2N HCl and 40 µl of plasma. After 30 min at room temperature, the mixtures were centrifuged. Non-protein radioactivity was counted in 40 µl of the supernatant fluid. Protein-bound radioactivity was calculated from the difference between total and non-protein radioactivity.

Statistical analysis

Results from n experiments were expressed as mean ± standard error (mean ± SEM). Student's t-test for unpaired samples was used for analysis, with a level of significance at $p < 0.05$.

A visual inspection of the labelled plasma concentration versus time in linear and semi-logarithmic scales shows a profile typical for a Michaelis-Menten elimination kinetic. This is the reason why the disappearance kinetics of ^{3}H ASOR were fitted to the model:

$$\frac{dy}{dt} = \frac{Vm\hat{y}}{K_M + \hat{y}}$$

where Vm is the maximum elimination rate and K_M is the concentration of ^{3}H ASOR corresponding to an elimination rate equal to half to Vm. The dispersion error on the measured concentrations was not fully known, so we decided to estimate the Vm and K_M, minimizing the log likelihood function:

$$L = Z \frac{(yi - \hat{y}i)^2}{Vi} + \lg Vi$$

where Vi is the error variance of the i-th observation yi.

Note that usually weighted least squares are appropriately used when it is assumed that the error variance is known up to a proportional factor.

In our case the error variance is not known, so according to Skeiner [10] the following variance model was assumed.

$$Vi = a\hat{y}^b$$

where a and b are variance model parameters. These parameters as well as Vm and K_M were estimated using the PHARM program [11].

Results

Evaluation of the orosomucoid level and the percentage of its desialylation in normal and diabetic rat sera

The orosomucoid content of normal rat sera was 152 ± 23 mg/l ($n = 8$) and 154 ± 24 mg/l ($n = 8$), by EID and RID respectively, whereas in diabetic rats the serum orosomucoid content was 379 ± 129 mg/l ($n = 7$) by both methods. Compared to normal rats the orosomucoid level was significantly higher ($p < 0.001$) in diabetic rats. In contrast, the percentage of underevaluation by EID relative to RID indicating the degree of desialylation of orosomucoid was found to be within the normal range in both normal and diabetic rats. No accumulation of partially desialylated orosomucoid occured in diabetic rat serum.

Relationship between dose and removal of ^{3}H asialo-orosomucoid in control and diabetic rats

In the first set of experiments, increasing quantities of ^{3}H ASOR were injected to find the dose at which the liver could no longer eliminate the protein in a matter of

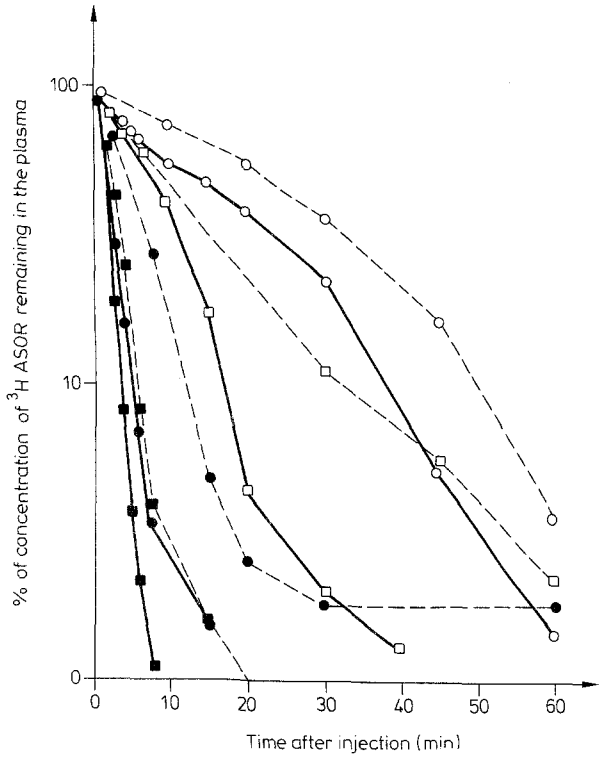

Fig. 1. Protein-bound radioactivity in the plasma of control rats (-) and diabetic rats (- - -) which received different quantities of ^{3}H asialo-orosomucoid (ASOR). The doses, expressed per 100 g body wt. were: 75 μg (■), 100 μg (●), 400 μg (□) and 1000 μg (○)

minutes. The behaviour of ^{3}H ASOR in plasma is illustrated with some representative curves of the protein-bound radioactivity as a function of the dose (Fig. 1). The 50% of ^{3}H ASOR remaining in blood was reached at later intervals as the dose of injected ^{3}H ASOR increased (e.g. with normal rats about 50% of injected cpm were present in blood at 2 min for 75 μg/100 g, 2 min 30 s for 100 μg/100 g, 8 min for 400 μg/100 g and 15 min for 1000 μg/100 g after injection). The kinetics of plasma disappearance of ^{3}H ASOR were dose-dependent. By administrating 1000 μg of ^{3}H ASOR/100 g of body weight in a single dose, we could see that the liver of normal rats maintained maximal disappearance rates for at least 20 min.

Plasma disappearance of ^{3}H asialo-orosomucoid in the presence of native orosomucoid

Unlabelled native (sialylated) orosomucoid (1000 μg) was simultaneously injected with 100 μg of ^{3}HASOR in normal and diabetic rats (data not shown). No decrease in the coefficient of extraction of the labelled asialoglycoprotein was observed in either group of rats. The lack of competition between ASOR and orosomucoid indicates that these glycoproteins were cleared by different processes in both normal [12] and diabetic rats.

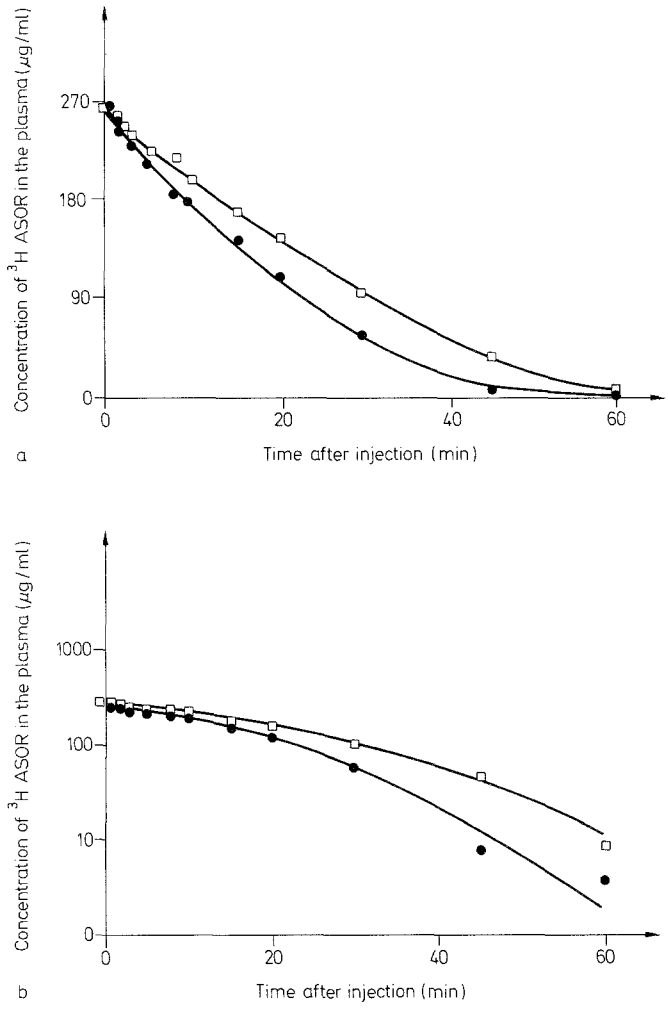

Fig. 2. Representative curves in linear (**a**) and logarithmic (**b**) scales of disappearance of the labelled protein from plasma of a control (●) and a diabetic rat (□). The dots are experimental dots and the lines are the result of the modeling according to the Michaelis-Menten equation

Plasma disappearance of ^{3}H asialo-orosomucoid in control and streptozotocin-diabetic rats

In this set of experiments a standard dose of 1000 μg ^{3}H ASOR per 100 g of body weight was administered to control ($n=7$) and streptozotocin-treated rats ($n=6$). The kinetic of disappearance was measured during 60 min. In Figure 2, the curves are plotted in linear and semi-logarithmic scales. The dots are experimental dots and the lines are the results of the modeling according to the Michaelis-Menten equation.

The kinetic analysis provided the final parameter estimates Vmax and K_M of ASOR removal from plasma in normal and diabetic rats (Table 1). ASOR was rapidly removed from bloodstream in normal rats, whereas in diabetic rats the asialoglycoprotein was cleared much more slowly. Mean values of Vmax = 9.60 ± 0.72 μg/min per 100 g body wt. and 6.70 ± 0.72 μg/min per 100 g body wt. were obtained for normal and streptozotocin-diabetic rats respectively, and were statistically signifi-

Table 1. Comparison of the maximal rates (Vmax) and the Michaelis constants (K_M) for the control and diabetic rats for our in vivo study and for our previous in vitro study [1, 12]

	In vivo studies		In vitro studies	
	Control rats ($n=7$)	Diabetic rats ($n=6$)	Control rats ($n=6$)	Diabetic rats ($n=6$)
Vmax (μg/min per 100 g body wt.)	9.60 ± 0.72[a]	6.70 ± 0.72[a]	0.81 ± 0.09[a]	0.32 ± 0.03[a]
K_M (nmol/l)	1.88 ± 0.31	1.44 ± 0.31	2.27 ± 0.43	2.32 ± 0.53

[a] $p < 0.05$

cant at $p < 0.05$. In contrast, the estimates of K_M, 1.88 ± 0.31 nmol/l and 1.44 ± 0.31 nmol/l for normal and diabetic rats respectively were not significantly different.

Phosphotungstic acid-nonprecipitable radioactivity was simultaneously evaluated and expressed as a percentage of the total radioactivity. Within the initial 30 min, no significant protein-unbound radioactivity was detected in the plasma of both normal and diabetic rats. The percentage then increased and reached 50–60% at 60 min and remained constant in both groups of rats. This finding seems to indicate that, after the uptake of ASOR by tissues [1, 12, 13], the intracellular catabolism of ASOR and the subsequent elimination of radiolabelled metabolites were not apparently altered in diabetic rats [4].

Discussion

The present study provides in vivo estimates of the kinetic parameters of ASOR removal from plasma of diabetic rats. The effects of insulin treatment were not studied here. In fact, we have previously shown that insulin therapy of streptozotocin-diabetic rats restored the ability of isolated hepatocytes to bind and take up ^{3}H ASOR, and that the withdrawal of insulin led again to a decreased binding and uptake of ^{3}H ASOR [3].

Several aspects of these in vivo studies are in agreement with our in vitro studies [3]. First, the maximal rate of removal of ASOR (Vmax = 6.70 μg/min per 100 g of body wt.) from plasma of diabetic rats was significantly lower than that of normal rats (Vmax = 9.60 μg/min per 100 g of body wt.). Second, no modification of Michaelis constant was noted in diabetic rats. These data could be explained either by the presence of desialylated orosomucoid in the plasma, which competes with ^{3}H ASOR, or by a decreased blood flow or by a decreased uptake of ASOR by some tissues, mainly the liver.

The first hypothesis is ruled out since, despite an increased amount of plasma orosomucoid which was previously described [5, 6], no asialo-orosomucoid was detected. The second explanation is not retained, with a decreased blood flow never being described in diabetic rats. The third suggestion is the most relevant, taking into account our previous data showing a decreased number of asialoglycoprotein receptors with diabetic rat hepatocytes. Since the major site of catabolism of injected asialoglycoproteins is the liver with the kidney and the gut participating to a much lower extent [12, 13, 14], it appears that the disappearance of ASOR from plasma approximately represents the capacity of liver to bind and internalize asialoglycoproteins.

In order to compare adequately the maximal rate of removal of ^{3}H ASOR from plasma to the maximal rate of endocytosis of ^{3}H ASOR by hepatocytes, the last parameter was expressed as 100 g of body weight. Assuming a rat liver is 1.6×10^8 hepatocytes/g and a standard weight for a liver of 250 g rats is 7 g, the maximal rates of endocytosis of ^{3}H ASOR become 0.80 ± 0.09 and 0.32 ± 0.03 μg/min per 100 g of body weight in normal and diabetic rats respectively, which is ten- to twenty-fold the maximal rate of removal of ^{3}H ASOR from plasma. This discrepancy between in vitro and in vivo studies was first described by Pardridge et al. [17], who suggested that, during the isolation procedure of hepatocytes, hepatic asialoglycoprotein receptors can undergo internalization.

In diabetic rats, the decreased rate of hepatic clearance of ^{3}H ASOR is not due to a reduced affinity of ASOR for its receptor but is rather related to a decreased number of functional receptors. Since the presence of sialic acid residues at the ultimate position of the receptor is needed to bind the galactose-terminated glycoproteins, an alteration of the carbohydrate moiety of membrane glycoprotein which occurs in experimental diabetes [18, 19, 20] could be evoked. We previously demonstrated that the impairment of the uptake of ASOR was not related to a decreased membrane sialic acid content [20]. On the other hand, the variations in the number of asialoglycoprotein receptors could be related to the regulatory mechanism of insulin on protein synthesis [21] and/or breakdown [22, 23]. Diabetes mellitus leads to a decreased rate of synthesis of extra and intrahepatic proteins as well as to an increased rate of protein catabolism.

The alterations in the number of asialoglycoprotein receptors we observed with the in vitro model are markedly confirmed by the present in vivo experiments.

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