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Differential Effect of Vanadate on Receptor-Mediated Endocytosis of the Asialoglycoprotein Receptor in Hepatocytes from Normal and Diabetic Rats

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ABSTRACT. Insulin-dependent diabetes has been shown to affect several aspects of receptor-mediated endocytosis. Since vanadate, a phosphate analogue, is known to exert insulin-like actions in target tissues, we studied the effects of vanadate on the endocytosis of the asialoglycoprotein receptor (ASGP-R) after its administration either *in vivo* (oral therapy) and/or *in vitro* by direct incubation of isolated hepatocytes with vanadate. The surface binding, internalization, and degradation of ^{3}H-asialoorosomucoid (^{3}H-ASOR), a prototype ligand of the ASGP-R, were decreased in diabetic rats by approximately 36.5%, 22.3%, and 12.9%, respectively. These values were normalized in diabetic rats treated by vanadate. Similarly, vanadate treatment normalized the biphasic dissociation of ^{3}H-ASOR/ASGP-R complexes by restoring the rapid dissociation process. In contrast, vanadate treatment did not affect any of these endocytic parameters in normal rats. *In vitro* experiments were monitored by direct incubation of isolated hepatocytes with 10 mM vanadate. This incubation created an inhibitory effect on the endocytic parameters. In this work, we have demonstrated that vanadate treatment can reverse the alterations induced by diabetes on receptor-mediated endocytosis of the ASGP-R. BIOCHEM PHARMACOL **54**;3:349–355, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. endocytosis; asialoglycoprotein receptor; diabetes; hepatocytes; vanadate

Vanadium is present in varying amounts in various tissue compartments of the body. The highest concentrations are seen in liver, kidney, and bones. Its role has long been elusive and controversial. The oxidized form, vanadate, is a phosphate analogue and has been shown to be a strong effector of ATPases involved in ion transport, tyrosine phosphatases, and adenylate cyclases (for review see [1]). The most intense area of research deals with the insulinomimetic effects of vanadate that could be proposed as a therapeutic means to correct some of the symptoms induced by diabetes (for review see [2]).

Receptor-mediated endocytosis is a critical process that allows the efficient and specific uptake of a variety of nutrients, hormones, and growth factors to transport them into the cell [3]. In our previous studies on receptor-mediated endocytosis of the asialoglycoprotein receptor (ASGP-R†) in rat hepatocytes, we showed in particular that ASGP-R endocytosis is deficient in hepatocytes isolated from rats suffering from diabetes induced by streptozotocin. Under these conditions, we demonstrated that insulin-deficient diabetes is associated with a reduced binding capacity of the ASGP-R [4, 5] and strongly impairs the degradation of its ligand [6, 7]. Others have shown that vanadate is able not only to reduce the number of binding sites of asialoglycoproteins (ASGP) at the cell surface of intact rat hepatocytes by 70% [8, 9] but also to inhibit their degradation by blocking the transfer to lysosomes [9]. It appears that several steps along the endocytic pathway of the ASGP-R are potentially controlled by the action of vanadate. For example, phosphorylation events were involved in the regulation of internalization, endosomal function, and targeting to lysosomes [10–14].

Because of these well-described effects, it was interesting to investigate whether vanadate would correct the modifications in receptor-mediated endocytosis observed in diabetes. For this purpose, we compared the effects of vanadate on endocytosis in normal and streptozotocin-induced diabetic rats *in vivo*, after oral administration, or *in vitro* after direct incubation of isolated hepatocytes with the drug. Our results clearly demonstrate that vanadate *in vivo* can correct most of the modifications induced by diabetes in the endocytosis of the ASGP-R.

MATERIALS AND METHODS

Materials

Sodium orthovanadate was obtained from Janssen (Geel, Belgium). Human orosomucoid (Sigma, St. Quentin Fallavier, France) was desialylated with agarose-immobilized

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† *Abbreviations:* ASGP, asialoglycoprotein; ASGP-R, asialoglycoprotein receptor; ASOR, asialoorosomucoid; PTA, phosphotungstic acid; and STZ, streptozotocin.

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neuraminidase type X-A (EC 3.2.1.18), and ^3H labeling was performed by reductive methylation according to Wilder *et al.* [15] with [^3H]KBH_4 (50–65 Ci/mmol; CEA, Saclay, France). The specific radioactivity was 820 cpm/ng asialoorosomucoid (ASOR). Collagenase (type IV), streptozotocin (STZ), bovine serum albumin, and reagents for hepatocyte incubation were from Sigma; digitonin was from Merck (Darmstadt, Germany).

Animals

Male Sprague-Dawley rats (180–200 g) from Charles River (St. Aubin-les-Elbeuf, France) were housed in wire-bottomed cages and maintained at 22 ± 2° in a room with a 12-h light-dark cycle. Water and standard laboratory chow were freely available throughout. Diabetes was induced as described in [16], after an overnight fast with free access to water, by injection into the tail vein of streptozotocin (65.4 mg/kg body wt), which was dissolved in 0.2 M citrate, 38.5 mM NaCl buffer, pH 4.6. Control rats were injected with buffer alone.

STZ-treated rats were considered diabetic when glycosuric and when blood glucose levels were above 25 mmol/L. After 11 days, the rats were sorted into four experimental groups: normal, vanadate-treated normal, diabetic, and vanadate-treated diabetic rats. Vanadate-treated animals ingested orthovanadate in drinking water at a dose of 12 to 24 mg/day for 12 days before killing.

Blood Parameters

Immediately before sacrifice, blood was collected from the abdominal aorta, and plasma was separated and stored at −40° for later measurement of glucose (glucose-oxidase-based auto analyzer) and insulin concentrations (RIA kit CIS, Institut Pasteur-Paris, France).

Preparation of Isolated Hepatocytes

Hepatocytes were isolated by collagenase perfusion as described previously [17, 18]. Briefly, the rat liver was injected *in situ* at 37° with calcium-free 10 mM HEPES, pH 7.5, followed by 0.2 mg/mL collagenase in HEPES buffer supplemented with 1.5% (w/v) BSA and 1.3 mM $CaCl_2$. After two centrifugations (50 g, 60 s), cell pellets were resuspended in ice-cold medium. Viability was measured by trypan blue exclusion and was at least 85–95%. Prior to all experiments, suspensions of freshly isolated hepatocytes (3×10^6/mL) were incubated in HTT buffer (30 mM HEPES, 30 mM TES, 36 mM TRICIN, 68 mM NaCl, 5 mM KCl, 2.4 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 0.64 mM $MgCl_2$, 0.7 mM Na_2SO_4, 2.5 mM $CaCl_2$, 0.1% (w/v) BSA, pH 7.4) at 37° for 25 min during continuous gyratory shaking at 60 rpm to allow redistribution of cryptic receptors [19]. *In vivo* experiments refer to those where hepatocytes were isolated after oral administration of vanadate, and *in vitro* conditions define experiments in which hepatocytes isolated from each group of rats were directly incubated with vanadate.

Endocytosis of ASGP-R

Hepatocytes were resuspended in HTT buffer. When indicated, vanadate was dissolved immediately in this HTT buffer and adjusted to pH 7.4 at a final concentration of 10 mM. Aspecific binding was determined in the presence of a 100-fold excess of unlabeled ASOR or by including 20 mM EDTA to control incubations. When indicated, cells were permeabilized by a 20-min incubation at 4° with 0.055% (w/v) digitonin as described in [20, 21].

BINDING CAPACITY OF CELL-SURFACE AND TOTAL RECEPTORS. Binding capacity was determined after a 90-min incubation of the cell suspension in HTT buffer at 4° with a saturating concentration of ^3H-ASOR (2 μg/mL) with (total receptor binding) or without (surface receptor binding) previous perforation of the cells by digitonin. Excess of ^3H-ASOR was eliminated by three washes (0.15 M NaCl/2.5 mM $CaCl_2$), and the radioactivity of the pellet was counted after centrifugation of the cells.

CONTINUOUS ENDOCYTOSIS OF ^3H-ASOR. The cell suspensions were incubated with ^3H-ASOR (2 μg/mL) at 37° for 60 min. At indicated times, an aliquot of 4 mL was withdrawn from the flask, diluted with 2 mL of ice-cold medium, and divided into four aliquots for duplicate determinations. All the following steps were carried out at 4°.

Treatment of the first aliquot. After three wash/centrifugation cycles (0.15 NaCl/2.5 mM $CaCl_2$), the cell pellet was resuspended in 0.15 mM NaCl/20 mM EDTA to remove surface-bound ^3H-ASOR. After 15 min of incubation at 4°, the amount of ligand bound to the cell surface was measured by the radioactivity of the supernatant after gentle centrifugation. The cell pellet was washed once more (0.15 mM NaCl/20 mM EDTA) and then counted to measure the total amount of intracellular ligand.

Treatment of the second aliquot. The total amount of degraded ^3H-ASOR (so-called phosphotungstic acid soluble) was measured by adding an equal volume of 2.5% (w/v) phosphotungstic acid (PTA) in 2 N HCl to the supernatant of hepatocytes perforated by digitonin for 30 min. After centrifugation, the radioactivity in the supernatant was counted and corresponded to the total amount of degraded ^3H-ASOR.

DISSOCIATION OF ASOR-RECEPTOR COMPLEXES DURING A SYNCHRONOUS WAVE OF LIGAND UPTAKE. Cells were allowed to bind ^3H-ASOR at 4° for 60 min and then were washed three times to eliminate the excess of unbound ligand. They were then resuspended in prewarmed buffer (37°) and kept in suspension in a gyratory shaking bath. At indicated times, 4 mL was withdrawn from the flask and monitored as described above.

TABLE 1. Effects of oral vanadate on body weight and blood assay parameters

	Normal	Vanadate-treated normal	Diabetic	Vanadate-treated diabetic
Weight gain (g/day)	9.81 ± 3.51	7.61 ± 3.06 NS	−1.80 ± 1.20***	−0.82 ± 0.90***
Glucose (mM)	10.10 ± 0.50	8.30 ± 1.00*	38.50 ± 5.90***	28.40 ± 5.40*** (++)
Insulin (mU/L)	30.00 ± 1.70	23.40 ± 5.50*	13.00 ± 3.60***	12.00 ± 4.20***

Glucose and insulin were measured as described in "Materials and Methods." Results are expressed as means ± SD for five animals. As compared with normal: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. As compared with diabetic: ++$p < 0.01$.

Treatment of the first aliquot. After centrifugation and two washes (0.15 M NaCl/20 mM EDTA) to remove the ligand bound to the surface, the amount of total intracellular ligand was measured by the radioactivity counted in the cell pellet.

Treatment of the second aliquot. After centrifugation and two washes (0.15 M NaCl/20 mM EDTA) to remove surface-bound ligand, the cell pellet was resuspended in 1 mL of HTT buffer with digitonin to permeabilize the cells. After 20 min, cells were centrifuged, and the unbound ligand was removed by discarding the supernatant. The cell pellet was washed twice (0.15 M NaCl/2.5 mM $CaCl_2$). The radioactivity of the pellet corresponded to the amount of the intracellular ligand that was still bound to the receptor. The dissociation of ^{3}H-ASOR from its receptor was then determined by the ratio of the amount of intracellular ligand bound to the receptor to the total amount of intracellular ligand.

RESULTS

Effects of Oral Administration of Vanadate on Weight and Serum Parameters

Weight gain in vanadate-treated and untreated normal rats was not significantly different (Table 1). The retardation of growth induced by diabetes could be stabilized but not normalized after vanadate treatment. Diabetes increased the concentration of blood glucose 3.8-fold and decreased the concentration of endogenous insulin by 57%. Vanadate treatment significantly corrected the hyperglycemia in diabetic rats by 26.2% but was unable to normalize the endogenous concentration of blood insulin.

Effects of Oral Administration of Vanadate on ASGP-R Endocytosis

As seen in Fig. 1, vanadate when given orally did not significantly affect the binding capacity of either surface or intracellular ASGP-R in normal rats. In contrast, vanadate was able to rescue the binding capacity in diabetic rats. In good agreement with what has already been described [4, 5], diabetes significantly decreased both surface and total binding sites of the ligand to 8.5 ± 1.4 and 27.6 ± 2.6 ng/10^6 cells, respectively, compared with 23.3 ± 1.7 and 67.5 ± 4.3 ng/10^6 cells in normal rats. Vanadate treatment increased these values to 17.6 ± 3.7 ng/10^6 at the surface and 49.2 ± 4.3 ng/10^6 cells for total receptors in hepatocytes from diabetic rats, which corresponds to 76% and 73% of the normal values, respectively. In four groups of hepatocytes, the distribution between intracellular and plasma membrane localization could not be modified either by diabetes or by vanadate treatment.

Internalization and degradation experiments were then performed at 37° in the presence of a continuous flux of ligand at a saturating concentration. In normal rats, the amount of ligand bound to intracellular receptors plateaued at 60 min independently of treatment by vanadate (Fig. 2) (120.4 ± 24.0 and 123.1 ± 27.1 ng/10^6 cells, respectively). This plateau was approximately five-fold higher than the amount of binding at the surface. In contrast, vanadate had a marked effect on diabetes, since the amount of internalized ligand, strongly reduced by the diabetic pathology (26.9 ± 5.7 ng/10^6 cells), could be rescued to levels close to physiology (105.5 ± 17.5 ng/10^6 cells).

After 60 min of continuous uptake, the degradation process was also affected, since the amount of degraded

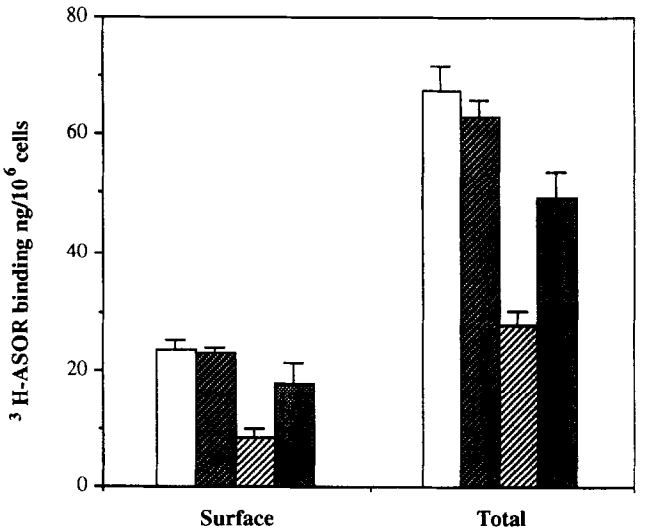

FIG. 1. Effects of oral vanadate on surface and total ASGP-R binding. ^{3}H-ASOR binding was determined by incubating hepatocytes isolated from normal (□), vanadate-treated normal (▨), diabetic (▨), and vanadate-treated diabetic (▨) rats at 4° for 90 min with (total binding) or without (surface binding) 0.055% (w/v) digitonin. Bars correspond to the average value from four experiments, and error bars represent standard deviations.

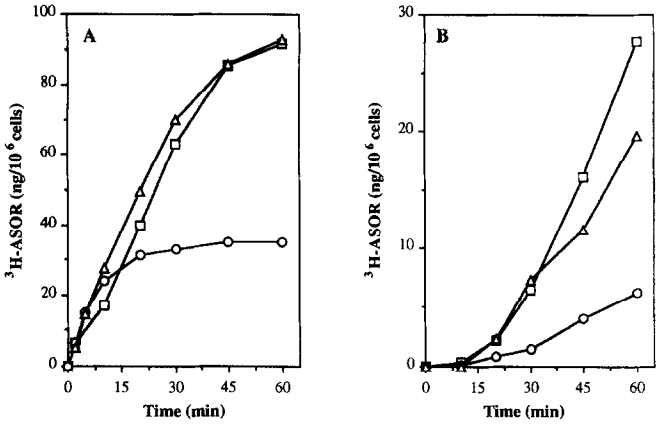

FIG. 2. Effects of oral vanadate on internalization and degradation. Hepatocytes (3 × 10^6 cells/mL) isolated from normal (□), diabetic (○), and vanadate-treated diabetic (△) rats were incubated at 37° for various times with ^{3}H-ASOR at saturating concentration (2 μg/mL). At the indicated times aliquots were removed, and the amounts of (A) internalized ligand and (B) phosphotungstic acid-soluble degraded ligand were determined as described in "Materials and Methods." Each point represents the mean of duplicate measurement. Similar results were obtained from four separate experiments.

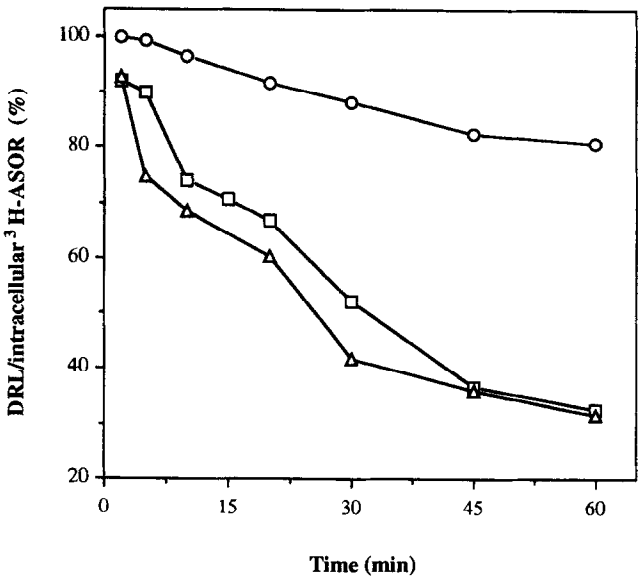

FIG. 3. Effects of oral vanadate on kinetics of dissociation of ligand/ASGP-R complexes during a synchronous wave of prebound ^{3}H-ASOR. Hepatocytes (3 × 10^6 cells/mL) isolated from normal (□), diabetic (○), and vanadate-treated diabetic (△) rats were preincubated at 4° for 1 h with ^{3}H-ASOR at saturating concentration (2 μg/mL), washed three times to remove unbound ligand, and then resuspended with prewarmed buffer at 37°. At given times, aliquots were removed and washed with 0.15 M NaCl + 0.02 M EDTA to release surface-bound ligand. Cells were then permeabilized by incubation with 0.055% (w/v) digitonin at 4° for 20 min and then washed twice with 0.1 M NaCl + 2.5 mM $CaCl_2$. The intracellular radioactivity determined in the presence (digitonin-resistant ligand (DRL)) and in the absence (total intracellular ligand) of digitonin was counted. The y axis represents the ratio of receptor-bound ligand to total intracellular ligand at the corresponding time. Data represent the average of duplicate determinations from one representative of three similar experiments.

ligand was eight-fold lower in diabetic rats as compared with control rats (3.2 ± 1.7 vs. 24.8 ± 3.3 ng/10^6 cells, respectively). Once again, vanadate treatment had no effect in normal rats but was able to normalize the amount of degraded ligand up to physiological values in diabetic rat hepatocytes (Fig. 2). The degradation of intracellular ligand is controlled by two critical steps, i.e. the dissociation of ligand-receptor complexes in the acidic lumen of endosomes and the migration of the dissociated ligand to the lysosome. Therefore, we wondered which of these steps was modified by diabetes and susceptible to be corrected by vanadate treatment.

Effect of Vanadate Treatment on Ligand Dissociation

To answer that question, we used the technique of a single wave of internalization. Endocytosis of a synchronous cohort of ligand-receptor complexes allows one to dissect biochemically, and more precisely, some of the endocytic steps such as internalization and the endosomal dissociation of the ligand from its receptor. The dissociation of ligand-receptor complexes was measured by the ratio of digitonin-resistant intracellular ^{3}H-ASOR (bound to receptors) vs. the total amount of intracellular ^{3}H-ASOR. The kinetic analysis of the experiment showed a biphasic dissociation in normal rats (Fig. 3) in good agreement with published results [18, 22]. Vanadate-treated rats presented a similar slope of dissociation, and most of the intracellular ligand was dissociated after 60 min in both normal and treated rats. In diabetic rats, the slow dissociation slope was, however, the only one present, while the rapid dissociation process disappeared. The dissociation was partial, reaching ca. 10% after 20 min. After 60 min, only 20% of the ligand initially bound to the cell surface could dissociate from its receptor. Vanadate treatment was fully efficient in restoring the biphasic slope with a rapid dissociation process (Fig. 3).

Effect of Vanadate Incubations on ASGP-R Endocytosis in Normal and Diabetic Rat Hepatocytes

In addition to its effects *in vivo* as described above, vanadate can also decrease ligand binding and degradation *in vitro* when it is incubated directly with hepatocytes of normal rats [8, 9]. It was therefore interesting to investigate the effect of an extra preincubation with vanadate on the different series of hepatocytes. Hepatocytes were incubated for 20 min at 37° with 10 mM vanadate *in vitro* before the binding capacity of the receptors was measured. A slight inhibitory effect was observed for each group. The decrease was similar for surface and total receptors in normal rats (73.3 ± 6.7% and 78.4 ± 7.4% of the control, respectively), suggesting that vanadate most likely inactivates rather than redistributes the receptors. The decrease was smaller in rats where vanadate had been given *in vivo* (90.5 ± 6.4% vs. 87.6 ± 3.7% of the controls, respectively), as if its maximal effect had already

TABLE 2. Effects of incubations with 10 mM vanadate on ASGP-R endocytosis parameters

	Normal	Vanadate-treated normal	Diabetic	Vanadate-treated diabetic
(1) Surface binding control (ng/10^6 cells)	23.3 ± 1.7	22.8 ± 0.9 (NS)	8.5 ± 1.4 (+++)	17.6 ± 3.7 (++)
(%/control)	73.3 ± 6.7 (**)	90.5 ± 6.4 (NS)	66.9 ± 8.7 (**)	75.8 ± 6.5 (**)
(1) Total binding control (ng/10^6 cells)	67.5 ± 4.3	63.1 ± 2.9 (NS)	27.6 ± 2.6 (+++)	49.2 ± 4.3 (+++)
(%/control)	78.4 ± 7.4 (*)	87.6 ± 3.7 (**)	85.4 ± 1.9 (***)	92.2 ± 1.9 (**)
(2) Internalization control (ng/10^6 cells)	120.4 ± 24.0	123.1 ± 27.1 (NS)	26.9 ± 5.7 (+++)	105.5 ± 17.5 (NS)
(%/control)	66.5 ± 3.7 (***)	64.1 ± 8.9 (**)	54.3 ± 7.1 (**)	40.7 ± 4.8 (***)
(2) Degradation control (ng/10^6 cells)	24.8 ± 3.3	34.6 ± 6.0 (+)	3.2 ± 1.7 (+++)	19.8 ± 6.7 (NS)
(%/control)	35.7 ± 9.2 (***)	30.7 ± 11.3 (**)	37.6 ± 7.5 (***)	37.3 ± 4.6 (***)

(1) Hepatocytes were preincubated at 37° for 20 min in the presence or absence (control) of 10 mM vanadate, and surface and total binding of ^{3}H-ASOR were performed at 4° as described in "Materials and Methods."

(2) Hepatocytes were incubated at 37° with ^{3}H-ASOR (2 μg/ml) for 60 min in the presence or absence (control) of 10 mM vanadate. The cells were transferred to 4°, and the amounts of ^{3}H-ASOR internalized and degraded were determined as described in "Materials and Methods."

All data are means ± SD from four independent experiments. As compared with normal: $+p < 0.05$, $++p < 0.01$, $+++p < 0.001$. As compared with 100%: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

been reached *in vivo*. In diabetic rats, however, the binding capacity was more reduced at the cell surface as compared with total receptors, whether the rats had been treated (75.8 ± 6.5% and 92.2 ± 1.9% of the controls, respectively) or not (66.9 ± 8.7% and 85.4 ± 1.9% of the controls, respectively). These results suggest that vanadate treatment allows the redistribution of the receptors toward intracellular compartments for both conditions of diabetic rats.

We then studied the effects on the next steps of endocytosis. When added to isolated hepatocytes from all groups of rats, vanadate showed a similar inhibitory effect on the internalization of the ligand and even a stronger inhibition on the degradation of the ligand (Table 2). When we used the single wave technique to follow the dissociation step, we were able to show that incubations with vanadate resulted in a partial inhibition of the dissociation step in normal rats and in treated diabetic rats. By contrast, vanadate incubations had no effect on diabetic rats (Fig. 4).

DISCUSSION

Many studies have shown that oral vanadate treatment in rats nearly normalizes the pathological effects induced by streptozotocin diabetes. It was suggested that vanadium compounds exert insulin-like actions. The urgent need of new molecules for oral insulinomimetic therapy should not, however, elude the necessity to characterize any candidate as thoroughly as possible to truly assess the benefits of these new treatments to help cure the cascade of complications induced by chronic diabetes. For instance, a recent work by Watkins *et al.* [23] reported that vanadate treatment was unable to reverse the increased biliary clearance of rose bengal in diabetic rats.

Our laboratory has substantial expertise in the hormonal regulation of receptor-mediated endocytosis. For example, we demonstrated that the endocytosis of the ASGP-R is partially inhibited in streptozotocin-induced diabetic rats [4–7, 24]. It was then possible to characterize the metabolic effects of vanadate on receptor-mediated endocytosis, a critical process involved in multiple cellular functions. The results reported in this manuscript clearly show that oral administration of vanadate to diabetic rats is beneficial and can restore the endocytic properties of the ASGP-R.

While no effect of vanadate could be shown on normal rats, thereby confirming its non-toxic nature, it could fully restore a proper endocytic function in diabetic rats, as evidenced by the correction to physiological levels of different endocytic parameters such as the binding of the ligand, its internalization and degradation, and the recovery of biphasic kinetics for the dissociation process. However, the mechanisms of action of vanadate as an insulinomimetic are not clear. In this study, the benefits of vanadate do not result from a rise in blood levels of insulin nor can they be explained by an antihyperglycemic action since the oral treatment, although decreasing blood glucose, could not eventually restore the glucose homeostasis. Because the decrease in insulin observed in diabetes correlates with a similar decrease in vanadium in liver [25] and insulinemia is not normalized by vanadate treatment, it could be speculated that vanadate treatment is able to restore the hepatic stock of vanadium. This restoration could then enhance the action of residual insulin and reverse the diabetic pattern of the ASGP-R. Alternatively, vanadate could affect other parameters such as tyrosine kinase activity on other receptors than the insulin receptor itself.

The effects of vanadate observed *in vivo* cannot be extrapolated *in vitro* and vice-versa, since the response of ASGP-R to vanadate appears to be dependent on the mode of administration; we observed a normalizing therapeutic effect in orally treated diabetic rats but an inhibitory effect when vanadate was directly added to hepatocytes isolated from the four series of rats. However, these contradictory effects could be explained by the experimental differences

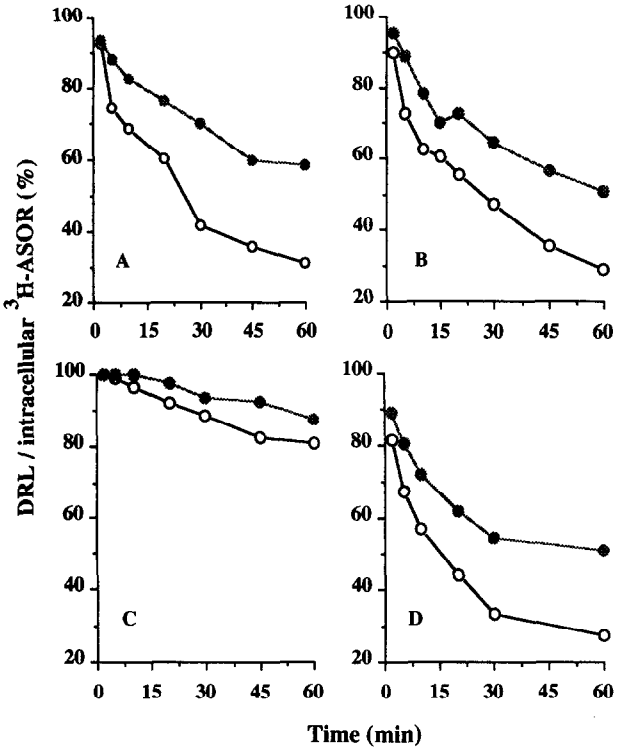

FIG. 4. Effects of incubations with 10 mM vanadate on kinetics of dissociation of ligand/ASGP-R complexes during a synchronous wave of prebound ^{3}H-ASOR. Hepatocytes (3 × 10^6 cells/mL) isolated from normal (A), vanadate-treated normal (B), diabetic (C), and vanadate-treated diabetic (D) rats were preincubated at 4° for 1 h with ^{3}H-ASOR at saturating concentration (2 μg/mL), washed three times to remove unbound ligand, and then resuspended with prewarmed buffer at 37° in the absence (open symbols) or presence (closed symbols) of 10 mM vanadate. At given times, aliquots were removed and washed with 0.15 M NaCl + 0.02 M EDTA to release surface-bound ligand. Cells were then permeabilized by incubation with 0.055% (w/v) digitonin at 4° for 20 min and then washed twice with 0.15 M NaCl + 2.5 mM $CaCl_2$. The intracellular radioactivity determined in the presence (digitonin-resistant ligand (DRL)) and in the absence (total intracellular ligand) of digitonin was counted. The y axis represents the ratio of receptor-bound ligand to total intracellular ligand at the corresponding time. Data represent the average of duplicate determinations from one representative of three similar experiments.

within the two protocols. This is true for the actual concentration of vanadate, which is different according to the means of administration. Oral treatment leads to an intrahepatic level of vanadate that is much lower than the levels obtained when the hepatocytes have been incubated directly with the drug *in vitro*. As a consequence, the concentration obtained *in vitro* can induce more pronounced effects than *in vivo*, even close to toxic levels. When vanadate is administered *in vivo*, it can reach the circulation and be distributed in several tissues, entering cells by anionic transport and exerting pleiotropic effects. The beneficial effects of vanadate in diabetes could result from a response of the liver to a chronic treatment of the whole organism, while incubations of hepatocytes result in a direct response of the hepatocytes without other interference. This could explain the different effects observed between *in vivo* and *in vitro* treatments.

It has been postulated that the insulin-mimetic effects of vanadate are mediated by the inhibition of enzymes involved in the transfer and release of phosphates. It is known that several steps in receptor-mediated endocytosis are regulated by phosphorylation events. It was reported that the ASGP-R is associated with a tyrosine kinase activity in HepG2 human hepatoma cells [26] and in rat hepatocytes [27]. Although the role of this activity remains obscure, tyrosine phosphorylation events could regulate the constitutive recycling of receptors [11] by a cycle of activation/inactivation of the receptors [27]. However, any other step could be regulated in a similar fashion.

Our results clearly demonstrate that the population of ASGP-R that is inactivated by diabetes fully recovers its endocytic properties after treatment by vanadate. The rapid phase of dissociation that disappears in diabetes is also normalized. The dissociation process depends on endosomal acidification, which is subject to multiple regulations. In addition to proton pumps, several factors can control the pH of endosomes: the entry of anions and the exit of cations as well as the passive dissipation of the proton gradient [28]. Because of its similarity to phosphate, vanadate could affect ATP-dependent regulations. The recovery of a normal biphasic dissociation curve in diabetic rats, after vanadate treatment, allows a degradation that is close to physiological values. This result confirms our previous study where the use of the carboxylic ionophore monensin led us to conclude that the marked decrease in ligand degradation observed in diabetes was a consequence of an impaired acidification [29]. The degradation was strongly decreased in all experimental groups of hepatocytes. This observation underlines the inhibition of access of the ligand to lysosomes under vanadate treatment, which confirms previous results in normal rats [8, 9]. Subcellular fractionation experiments have shown that vanadate inhibits the degradation of ASGP by preventing its access to lysosomes [9].

In conclusion, we have shown that vanadate contributes to correcting endocytic processes that are strongly deficient in diabetes. Further research is clearly needed to define the precise mechanisms of this class of potential therapeutic agents and provide critical data to find a cure for diabetes.

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