

Treatment of streptozotocin-induced diabetic rats with vanadate and phlorizin prevents the over-expression of the liver insulin receptor gene

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Abstract

Administration of vanadate, an insulinomimetic agent, has been shown to normalize the increased number of insulin receptors in the liver of streptozotocin-induced diabetic rats. In the present study, the effects of vanadate on various steps of expression of the liver insulin receptor gene in diabetic rats have been analyzed and compared with those of phlorizin, a glucopenic drug devoid of insulinomimetic properties.

Livers of rats killed 23 days after streptozotocin injection showed a 30–40% increase in the number of cell surface and intracellular insulin receptors, a 50–90% increase in the levels of 9.5 and 7.5 kb insulin receptor mRNA species, and a 20% decrease in the relative abundance of the A (exon 11[−]) insulin receptor mRNA isotype. Daily administration of vanadate or phlorizin from day 5 to day 23 prevented the increase in insulin receptor number and mRNA level, and vanadate treatment also normalized receptor mRNA isotype expression.

Unlike observations *in vivo*, vanadate and phlorizin differentially affected the expression of the insulin receptor gene in Fao hepatoma cells. Vanadate treatment (0.5 mmol/l for 4 h) decreased the levels of the 9.5 and 7.5 kb insulin receptor transcripts by at least twofold, without affecting the relative abundance of the A insulin receptor mRNA isotype. In contrast, phlorizin treatment (5 mmol/l for 4 h) slightly increased or did not affect the levels of the 9.5 and 7.5 kb insulin receptor transcripts respectively, and increased by twofold the relative expression of the A insulin receptor mRNA isotype.

It is suggested that, although mediated in part by a reversal of hyperglycemia, normalization of liver insulin receptor gene expression by vanadate treatment in diabetic rats may also involve a direct inhibitory effect of this drug on gene expression.

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Introduction

Vanadate treatment has been shown to correct a number of abnormalities in streptozotocin-induced diabetic rats (1–3), such as the altered expression of hepatic genes and corresponding products. These include enzymes involved in gluconeogenesis (4–8), glycolysis (4–8) and lipogenesis (9), the glucose transporter Glut 2 (8), serum albumin (10), and the transcription factors hepatocyte nuclear factor 1 (HNF1) (10), CAAT/enhancer binding protein α (7) and CAAT/enhancer binding protein β (11). However, partial correction of the altered expression of glycolytic and neoglucogenic enzymes (12), albumin (10) and HNF1 (10) also occurred after administration of phlorizin, which corrects the hyperglycemia of diabetic rats by inhibiting the renal tubular reabsorption of glucose (13). These findings suggest that, although vanadate can alter the expression of enzymes of carbohydrate

metabolism in cultured cells (14, 15), its *in vivo* effects on diabetic rats may be mediated in part by correction of hyperglycemia.

Streptozotocin-induced diabetic rats display an increase in liver insulin receptor number (16–18) and insulin receptor mRNA level (19–21) which is reversed by insulin treatment. Although evidence has been presented that vanadate treatment of diabetic rats normalizes insulin receptor number (22, 23), it is unknown whether such treatment also normalizes insulin receptor mRNA level. It is also unclear whether vanadate acts directly on insulin receptor gene expression, as is suggested by its ability to affect receptor number (24–28) and to decrease receptor mRNA level (28) in isolated cell systems. To address these questions, the *in vivo* effects of vanadate and phlorizin on liver insulin receptor number, insulin receptor mRNA level and insulin receptor mRNA isotype expression in streptozotocin-induced diabetic rats have been

comparatively examined. In addition, the effects of these drugs on insulin receptor mRNA level and insulin receptor mRNA isotype expression in Fao rat hepatoma cells have been studied.

Materials and methods

Animals and experimental design

Animal studies were conducted as described previously (8, 9) in accordance with the French guidelines for the care and use of experimental animals. Seven-week-old male Wistar rats (IFFA Credo, l'Arbresle, France) were divided into four groups: control rats ($n = 12$), rats rendered diabetic by a single injection of streptozotocin (45 mg/kg; $n = 14$), and diabetic rats treated with vanadate ($n = 9$) or phlorizin ($n = 8$), starting 5 days after streptozotocin injection (Upjohn, Kalamazoo, MI, USA). Vanadate (Sigma/Aldrich, St Quentin-Fallavier, France) was given daily as NaVO_3 in the drinking water (0.4 mg/ml). Phlorizin (Sigma/Aldrich) (0.4 g/kg body weight per day) was given for 3 days as two subcutaneous injections and then as a continuous subcutaneous infusion via an osmotic mini-pump. After 18 days of treatment (day 23), rats were killed and the liver was quickly removed. One portion was frozen in liquid nitrogen and stored at -80°C for RNA extraction; the remainder was immediately used for cell fractionation.

Preparation of liver subcellular fractions and assay of insulin binding activity

Liver total particulate, plasma membrane and Golgi-endosomal fractions were prepared and assayed for 5' nucleotidase, galactosyltransferase and insulin binding activity as described by Lopez *et al.* (18).

Liver RNA isolation and Northern blot analysis of insulin receptor mRNA

Liver RNA was extracted as described by Chomczynski & Sacchi (29). After denaturation with formaldehyde, it was size-fractionated on 1% agarose gels, transferred to a Hybond N membrane (Amersham, Les Ulis, France) and crosslinked by baking. Blots were hybridized with a ^{32}P -labeled insulin receptor antisense RNA probe, prepared by *in vitro* transcription of rat insulin receptor cDNA (a gift of Dr B Goldstein, Thomas Jefferson University, Philadelphia, PA, USA) as described previously (19). Hybridization signals were identified by autoradiography and quantitated by scanning densitometry (Hewlett Packard ScanJet II scanner connected to a MacIntosh computer). To correct for possible variations in the amount of RNA transferred to the membrane, blots were hybridized with a ^{32}P -labeled cDNA fragment coding for human poly A binding protein (pABP) (a gift from Dr T Grange, Institut Jacques Monod, Paris, France).

Analysis of liver insulin receptor mRNA isotypes

This was achieved by reverse transcription of RNA followed by radioactive PCR amplification of insulin receptor cDNA as described previously (28). As primers, oligonucleotides (Genset, Paris, France) spanning sequences 2530–2550 (sense primer) and 2822–2843 (antisense primer) were used; these primers generate 313 and 277 bp products encoding the B and A isoforms of the insulin receptor respectively. Products were separated by 8% polyacrylamide gel electrophoresis, visualized by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics, Bondoufle, France). Only the relative expression of the A and B insulin receptor mRNA isotypes, and not their actual concentrations, is given by this procedure.

Analysis of plasma glucose and plasma insulin

Plasma glucose was measured using the glucose oxidase method (Peridochrom, Boehringer, Mannheim, Germany) and plasma insulin was measured by RIA using a commercial kit (Oris Industrie, Gif sur Yvette, France).

Analysis of insulin receptor mRNA level and isotypes in rat hepatoma cells

Fao rat hepatoma cells (30) were cultured in Coon's modified Ham's F12 medium supplemented with 5% calf serum as recently described (28). At subconfluence, the medium was changed to a serum-free Ham's medium and 24 h later cells were treated with vanadate or phlorizin as indicated in legend to Fig. 4. Insulin receptor mRNA was analyzed by Northern blotting of total cellular RNA as described above for rat liver except that a ^{32}P -labeled insulin receptor cDNA probe was used for hybridization (28) and that insulin receptor signals were normalized to 18S rRNA signals as visualized by UV light after ethidium bromide staining. A and B insulin receptor mRNA isotypes were analyzed as described above.

Statistical analysis

Results are expressed as the mean \pm S.E.M. Comparisons between experimental groups were performed by analysis of variance followed by the Fisher, Bonferroni/Dunn and/or Kruskal–Wallis tests. P values < 0.05 were considered significant.

Results

Body weight, plasma glucose and plasma insulin concentrations

Eighteen days after streptozotocin injection, diabetic rats showed a reduced body weight relative to control,

Table 1 Plasma glucose and insulin levels in control (C), untreated diabetic (D), vanadate-treated diabetic (V) and phlorizin-treated diabetic (P) rats. Measurements were carried out after 18 days of treatment. Values, taken in part from Brichard *et al.* (8, 12), are the means \pm S.E.M. of 8–17 determinations within each group.

	C	D	V	P
Body weight (g)	320 \pm 6**	267 \pm 8	256 \pm 7	280 \pm 6
Plasma glucose (mmol/l)	7.2 \pm 0.1**	29.0 \pm 1.3	9.0 \pm 0.7**	9.7 \pm 0.4**
Plasma insulin (ng/ml)	1.74 \pm 0.11**	0.76 \pm 0.04	1.09 \pm 0.09	0.98 \pm 0.09

** $P < 0.01$ compared with untreated diabetic rats (Fisher test).

saline-injected rats. Diabetic rats also showed a fourfold increase in plasma glucose levels and a twofold decrease in plasma insulin levels relative to control animals (Table 1). Treatment of diabetic rats with vanadate or phlorizin caused almost complete normalization of plasma glucose levels, but did not significantly affect body weight and plasma insulin levels, which remained below the values observed in control rats (Table 1).

Insulin binding to liver subcellular fractions

Since changes in cell membrane composition have been reported in streptozotocin-induced diabetic rats (16, 18, 31), liver plasma membrane and Golgi-endosomal fractions were analyzed for 5' nucleotidase, a plasma membrane marker, and galactosyltransferase, a Golgi marker, respectively (Table 2). A moderate, 20% decrease in the specific activities of these enzymes was observed in cell fractions from untreated diabetic rats relative to other experimental groups. However, relative enrichments did not significantly differ between control, untreated diabetic, and vanadate- or phlorizin-treated diabetic rats.

Cell fractions were then tested for their ability to bind subsaturating concentrations of insulin (Fig. 1). In agreement with a previous study (18), plasma membrane and Golgi-endosomal fractions from control rats were about six- and threefold enriched in insulin binding activity relative to the total particulate fraction respectively. Induction of diabetes led to a moderate but significant increase in insulin binding activity, which was slightly greater in the Golgi-endosomal fraction (40% change) than in the total particulate and plasma

membrane fractions (25–30% change). Vanadate treatment of diabetic rats lowered insulin binding activity in each of these fractions to levels which did not significantly differ from those observed in control rats. Phlorizin treatment also decreased insulin binding activity, with full normalization of activity in the total particulate and plasma membrane fractions. Scatchard plots constructed from competitive inhibition curves with native insulin showed that the increase in insulin binding activity in cell fractions of untreated diabetic rats relative to other groups resulted solely from a change in receptor number (results not shown).

Liver insulin receptor mRNA level

Northern blot analysis of liver RNA using an insulin receptor RNA probe showed two major transcripts of 9.5 and 7.5 kb, a pattern typical of rodent species (Fig. 2). Densitometric quantitation of these transcripts using pABP as a standard showed that the 9.5 kb transcript was about four times more abundant than the 7.5 kb transcript, in agreement with previous reports (19, 20). Induction of diabetes led to a significant increase in the abundance of the two receptor transcripts, which affected the 7.5 kb transcript to a greater extent than the 9.5 kb transcript (90 and 50% change respectively). Vanadate and phlorizin treatments reduced the levels of the two transcripts to values which did not significantly differ from those observed in control rats. Although vanadate was slightly more effective than phlorizin in normalizing the 7.5 kb transcript, the difference between these two groups was not statistically significant.

Table 2 5' Nucleotidase and galactosyltransferase activities in liver plasma membrane (PM) and Golgi-endosomal (GE) fractions from control (C), untreated diabetic (D), vanadate-treated diabetic (V) and phlorizin-treated diabetic (P) rats. Results (means \pm S.E.M. of 4–11 determinations) are expressed as specific activity (in pmol/min/mg protein) and relative specific activity (RSA; ratio of specific activity in the PM or GE fraction to specific activity in the total particulate fraction).

Cell fraction	Enzyme	C	D	V	P
PM	5'-Nucleotidase				
	Specific activity	478 \pm 26**	384 \pm 20	481 \pm 25**	515 \pm 31**
	RSA	9.2 \pm 0.6	9.4 \pm 0.7	8.5 \pm 0.3	9.6 \pm 0.8
GE	Galactosyltransferase				
	Specific activity	568 \pm 35**	449 \pm 32	644 \pm 57**	497 \pm 24
	RSA	14.8 \pm 1.1	15.7 \pm 1.0	17.2 \pm 1.4	12.9 \pm 0.7

** $P < 0.01$ compared with untreated diabetic rats (Fisher test).

Liver insulin receptor mRNA isotype expression

Analysis of the products generated from insulin receptor mRNA by reverse transcription and radioactive PCR amplification revealed two components of 313 bp and 277 bp, which corresponded to the B (exon 11⁺) and A (exon 11⁻) isoforms of the receptor respectively (Fig. 3). Quantitation of these products showed that the B mRNA receptor isotype was the major isotype expressed, with the A isoform accounting for only 5–6% of the total

products. Streptozotocin treatment led to a slight, albeit statistically significant, decrease in the relative expression of the A isotype, which was reversed by vanadate but not by phlorizin treatment.

Comparative effects of vanadate and phlorizin on insulin receptor mRNA level and isoforms in rat hepatoma cells

The effects of vanadate and phlorizin on insulin receptor mRNA were comparatively studied in Fao rat hepatoma cells, a well-differentiated insulin-responsive hepatoma cell line (Fig. 4). In agreement with a recent report (28), treatment of these cells with 0.5 mM vanadate for 4 h led to a marked decrease in the relative abundance of the 9.5 and 7.5 kb insulin receptor mRNA species compared with 18S rRNA. In contrast, treatment of Fao cells with 5 mM phlorizin for 4 h did not affect the level of the 7.5 kb transcript and caused a weak, albeit significant, increase in the level of the 9.5 kb transcript.

To determine potential effects of vanadate and phlorizin on the relative expression of the A and B insulin receptor mRNA isoforms, total RNA from untreated and treated cells was extracted and subjected to reverse transcription and radioactive PCR amplification as described above (Fig. 5). In untreated cells, the B insulin receptor mRNA isotype was predominant, accounting for about 73% of total products. As previously observed after a 24-h treatment (28), a 4-h vanadate treatment did not affect the relative expression of the A and B insulin receptor mRNA isoforms. In contrast, phlorizin treatment led to a significant increase in the relative expression of the A mRNA isotype (from 27 to 47% of total products).

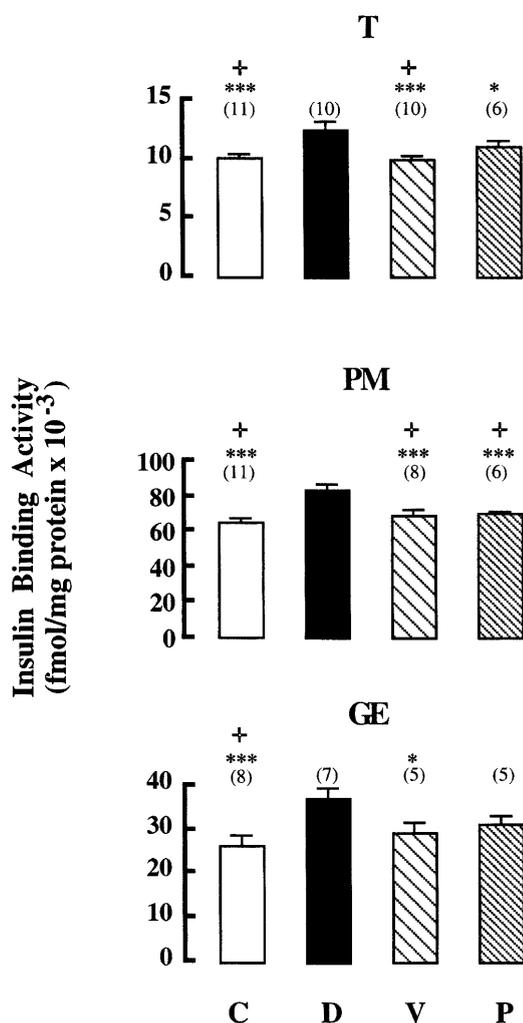


Figure 1 Insulin binding activity in liver subcellular fractions from control (C), untreated diabetic (D), vanadate-treated diabetic (V) and phlorizin-treated diabetic (P) rats. Liver total particulate (T), plasma membrane (PM) and Golgi-endosomal (GE) fractions were prepared and examined for specific insulin binding (ligand concentration 0.3 nmol/l). Each bar is the mean \pm S.E.M. of 5–11 determinations (shown in parentheses) on subcellular fractions originating from separate livers. Statistically significant differences vs untreated diabetic rats are indicated by asterisks (*) for the Fisher test (* P < 0.05; *** P < 0.001) and by crosses (+) for the Bonferroni/Dunn test (+ P < 0.05).

Discussion

Treatments with vanadate (4–11) and, to some extent, phlorizin (10, 12), have been shown to reverse the altered expression of a number of hepatic genes in streptozotocin-induced diabetic rats. Here, we have extended these findings to the insulin receptor gene, which is over-expressed in diabetic rats. However, despite similar *in vivo* effects of vanadate and phlorizin on insulin receptor expression, only the former decreased the insulin receptor mRNA level in rat hepatoma cells. These findings suggest that, although mediated in part by a reversal of hyperglycemia, normalization of liver insulin receptor gene expression by vanadate in diabetic rats may also involve a direct inhibitory effect of this drug on gene expression.

The ability of orally administered vanadate to reverse the increased insulin binding activity of crude liver membranes from diabetic rats was first reported by Meyerovitch *et al.* (22). Vanadate treatment has also been shown to lower insulin binding activity in wheat germ agglutinin-purified insulin receptor preparations from diabetic rats undergoing an hyperglycemic clamp

(23). In the present study, vanadate treatment fully normalized liver insulin binding activity in total cellular, cell surface and intracellular membranes. The last observation makes an effect of vanadate on insulin receptor trafficking such as that described in human IM9

lymphocytes (27) unlikely. Furthermore, vanadate treatment fully normalized the insulin receptor mRNA level, indicating that its *in vivo* effect on the expression of the insulin receptor gene, as with other genes (4–11), occurs at a pretranslational step.

In previous studies, phlorizin treatment of streptozotocin-induced diabetic rats has been shown to normalize the altered expression of the liver phosphoenolpyruvate carboxykinase (12), Glut 2 (12) and albumin (10) genes, but, unlike vanadate (8), it did not correct the lowered expression of the pyruvate kinase gene (12). In the present study, vanadate and phlorizin treatments were almost equally effective in their ability to prevent the increase in liver insulin receptor number and insulin receptor mRNA level observed in diabetic rats. While this study was in progress, phlorizin treatment was also shown to normalize the liver insulin receptor mRNA level in diabetic pregnant female rats, an animal model of insulin resistance (32). At a first glance, these *in vivo* findings suggest that hyperglycemia contributes to the over-expression of the liver insulin receptor gene and that vanadate may normalize receptor expression by decreasing plasma glucose. However, an increase in liver insulin receptor mRNA level has been observed both in hyperglycemic rodents, such as glucose-infused pregnant female rats (31) and genetically obese mice (33), and in hypoglycemic fasted rats (19, 21). In addition, although reported to induce an increase in insulin receptor mRNA concentration in HepG2 hepatoma cells (34) and 3T3 fibroblasts over-expressing the human insulin receptor (35), high glucose levels did not significantly affect this mRNA in primary cultures of rat hepatocytes (C Mrejen & S Hauguel-de Mouzon, personal communication) and Fao hepatoma cells (28). Thus, vanadate and phlorizin treatments could lower insulin receptor gene expression in diabetic rats by reversing abnormalities other than hyperglycemia – perhaps hyperglucagonemia, as has been suggested for enzymes involved in carbohydrate metabolism (8, 12).

The ability of vanadate and phlorizin to prevent the over-expression of the insulin receptor gene in diabetic rats in an identical fashion does not rule out additional, direct effects of vanadate on the expression of this gene. Indeed, vanadate has previously been shown to increase

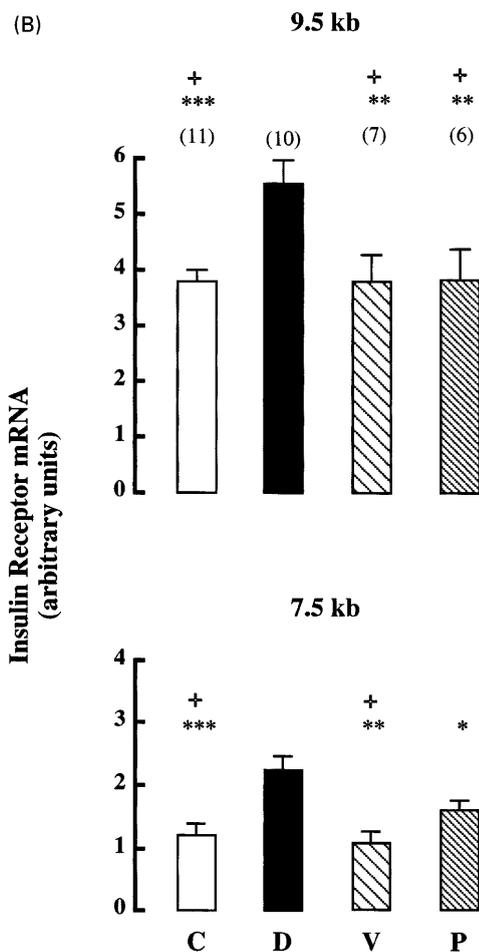
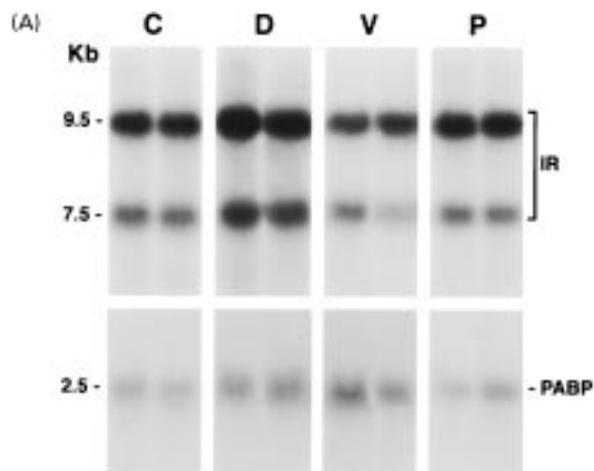


Figure 2 Northern blot analysis of liver RNA of control (C), untreated diabetic (D), vanadate-treated diabetic (V) and phlorizin-treated diabetic (P) rats. (A) Liver RNA was subjected to Northern blotting and hybridized with α -³²P-labeled insulin receptor (IR) and pABP probes as described in Materials and Methods. Within each experimental group, two representative experiments are shown. (B) The 9.5 kb and 7.5 kb insulin receptor transcripts identified on the autoradiograms were quantitated by scanning densitometry and results were normalized for pABP mRNA. Each bar is the mean \pm S.E.M. of 6–11 determinations (shown in parentheses) on RNA samples originating from separate livers. Statistically significant differences vs untreated diabetic rats are indicated by asterisks (*) for the Fisher test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001) and by crosses (+) for the Bonferroni/Dunn test (**P* < 0.05).

insulin receptor affinity (24) and to stimulate insulin-induced receptor down-regulation (25) in freshly isolated adipocytes, to decrease cell surface receptor number in cultured adipocytes (26), to decrease cell surface receptor number but increase total cellular receptor number, by inhibiting receptor degradation, in human IM9 lymphocytes (27), and to decrease total cellular receptor number, insulin receptor mRNA level and insulin receptor gene transcriptional activity in rat

Fao hepatoma cells (28). In the present study, the ability of vanadate to lower insulin receptor mRNA concentration in rat hepatoma cells has been confirmed, while phlorizin did not elicit such an effect. Thus, like the pyruvate kinase (14) and phosphoenolpyruvate carboxykinase (15) genes, the insulin receptor gene can be directly regulated by vanadate. Moreover, the effects of vanadate on these genes *in vitro* are consistent with those observed *in vivo* in diabetic rats. It should be noted, however, that the effective concentrations of vanadate in the medium of cultured cells (25–500 μM) are above those achieved in the serum of vanadate-treated diabetic rats (5–10 μM) (1–3).

It has been previously shown that, of the two alternatively spliced isoforms of insulin receptor mRNA, the B (exon 11⁺) isoform is predominantly expressed in liver, with no statistically significant difference between normal and diabetic rats (21). Our studies confirm that the B isoform predominates, but show that the A/B ratio is significantly decreased in diabetic rats. This change would be consistent with the increased expression of the A isoform reported in insulin-treated Fao hepatoma cells (28) and in liver and skeletal muscle from hyperinsulinemic obese diabetic Rhesus monkeys (36). Despite similar effects of vanadate and phlorizin on total insulin receptor mRNA, only vanadate normalized the relative expression of insulin receptor mRNA isoforms. As recently reported (28), the B isoform was also predominantly expressed in Fao hepatoma cells, albeit to a lesser degree than in normal liver. Surprisingly, however, the relative expression of the A isoform in Fao cells was unaffected by vanadate and markedly increased by phlorizin. The reasons for the discrepancies between the *in vivo* and *in vitro* effects of these drugs on insulin receptor mRNA isoforms are at present unknown.

In summary, both vanadate and phlorizin treatments prevent an increase in insulin receptor and insulin receptor mRNA concentrations in the liver of streptozotocin-induced diabetic rats, whereas only vanadate decreases insulin receptor mRNA concentration in rat

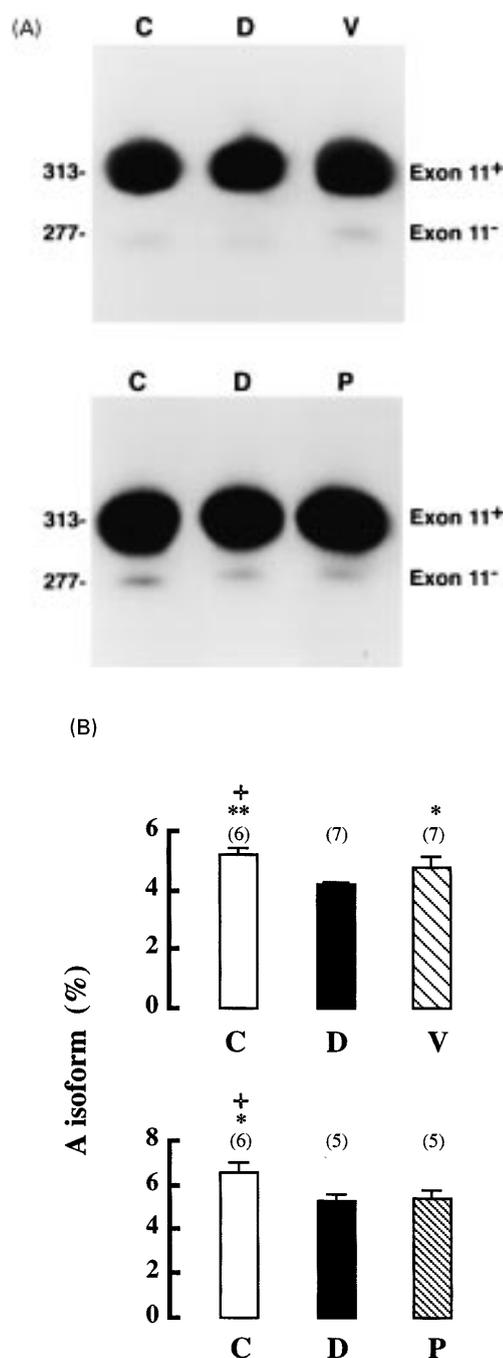


Figure 3 Relative expression of the A and B insulin receptor mRNA isoforms in control (C), untreated diabetic (D), vanadate-treated diabetic (V) and phlorizin-treated diabetic (P) rats. Partial sequences of insulin receptor cDNA either including exon 11 or not were generated from liver RNA by reverse transcription and radioactive PCR amplification as described in Materials and methods. (A) Amplification products corresponding to the exon 11⁻ (277 bp) and exon 11⁺ (313 bp) insulin receptor isoforms were resolved by electrophoresis and visualized by radioautography. (B) Products were quantitated using a PhosphorImager and the relative expression of the exon 11⁻ product (A isoform) was expressed as the percentage of total products. Each bar is the mean \pm S.E.M. of 5–7 determinations (shown in parentheses) on RNA samples originating from separate livers. Statistically significant differences vs untreated diabetic rats are indicated by asterisks for the Fisher test (* $P < 0.05$; ** $P < 0.01$) and by crosses (†) for the Bonferroni/Dunn test († $P < 0.05$).

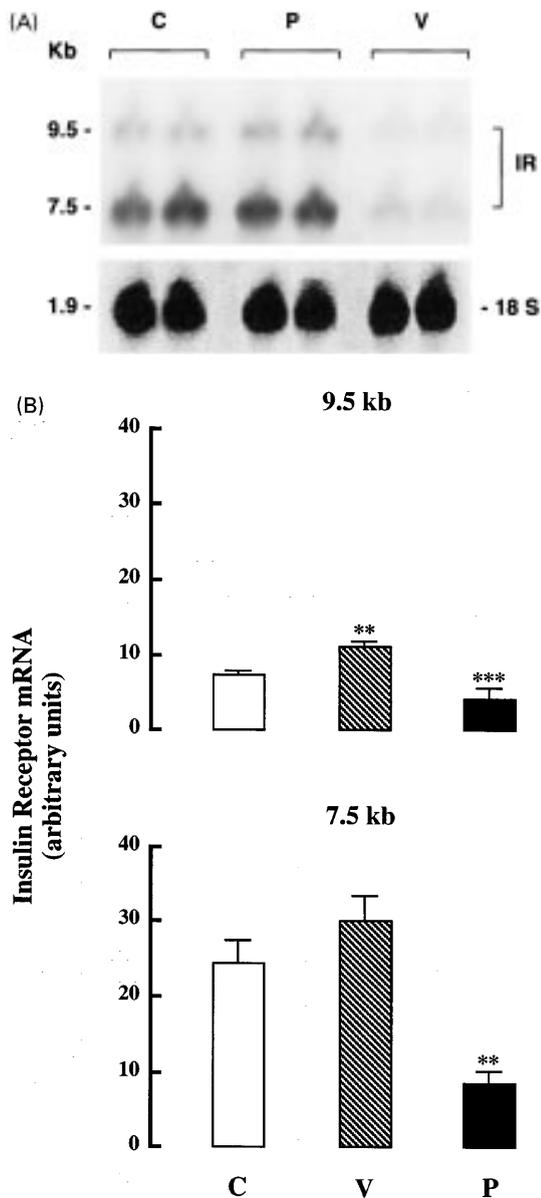


Figure 4 Northern blot analysis of insulin receptor mRNA in control (C), vanadate-treated (V) and phlorizin-treated (P) Fao rat hepatoma cells. (A) Cells were treated with 0.5 mmol/l vanadate or 5 mmol/l phlorizin or left untreated for 4 h. Total RNA (20 µg per lane) was subjected to Northern blotting and hybridized with a radiolabeled insulin receptor cDNA probe (IR) as described in Materials and methods. The results shown are representative of at least five independent experiments. (B) The 9.5 and 7.5 kb insulin receptor transcripts were analyzed by scanning densitometry with results normalized to 18S rRNA. Each bar is the mean ± s.e.m. of at least 5 independent determinations. Asterisks indicate a statistically significant difference vs control cells for the Kruskal–Wallis test (** $P < 0.01$; *** $P < 0.001$).

hepatoma cells. These findings suggest that two mechanisms may account for the ability of vanadate treatment to prevent the over-expression of the liver insulin receptor gene in diabetic rats: on the one hand, a

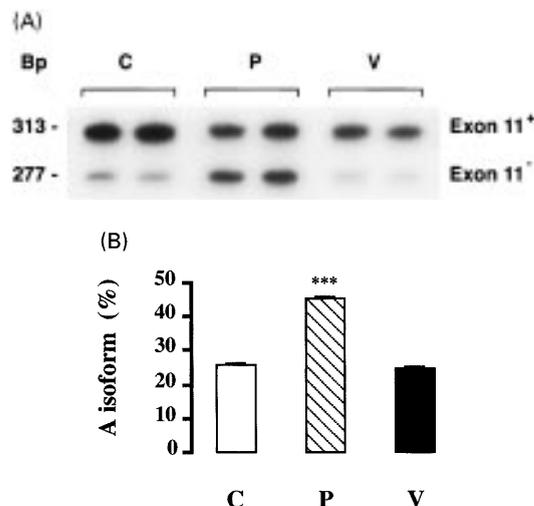


Figure 5 Relative expression of the A and B insulin receptor mRNA isotypes in control (C), vanadate-treated (V) and phlorizin-treated (P) Fao rat hepatoma cells. Total RNA was prepared from Fao cells that had been treated with 0.5 mmol/l vanadate or 5 mmol/l phlorizin or left untreated for 4 h. Partial sequences of insulin receptor cDNA either including exon 11 or not were generated as described in Materials and methods and legend to Fig. 3. (A) Amplification products corresponding to the exon 11⁻ (277 bp) and exon 11⁺ (313 bp) insulin receptor isotypes were resolved by electrophoresis and visualized by radioautography. (B) Products were quantitated using a PhosphorImager and the relative expression of the exon 11⁻ product (A isoform) was expressed as the percentage of total products. Each bar is the mean ± s.e.m. of 5 separate determinations. Asterisks indicate results significantly different from control cells for the Fisher test and the Bonferroni/Dunn test (*** $P < 0.001$).

correction of hyperglycemia and/or other metabolic abnormalities, yet to be identified, and on the other hand, a direct inhibitory effect of this drug on gene expression.

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