

Improvement of glucose homeostasis and hepatic insulin resistance in *ob/ob* mice given oral molybdate

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Abstract

Molybdate (Mo) exerts insulinomimetic effects *in vitro*. In this study, we evaluated whether Mo can improve glucose homeostasis in genetically obese, insulin-resistant *ob/ob* mice. Oral administration of Mo (174 mg/kg molybdenum element) for 7 weeks did not affect body weight, but decreased the hyperglycaemia (~20 mM) of obese mice to the levels of lean (L) (+/+) mice, and reduced the hyperinsulinaemia to one-sixth of pretreatment levels. Tolerance to oral glucose was improved: total glucose area was 30% lower in Mo-treated mice than in untreated *ob/ob* mice (O), while the total insulin area was halved. Hepatic glucokinase (GK) mRNA level and activity were unchanged in O mice compared with L mice, but the mRNA level and activity of L-type pyruvate kinase (L-PK) were increased in O mice by 3.5- and 1.7-fold respectively. Mo treatment increased GK mRNA levels and activity (by ~2.2-fold and 61% compared with O values), and had no, or only a mild, effect on the already increased L-PK variables. mRNA levels and activity of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) were augmented in O liver (sixfold and

by 57% respectively), and these were reduced by Mo treatment. Insulin binding to partially purified receptors from liver was reduced in O mice and restored by Mo treatment. Despite this correction, overall receptor tyrosine kinase activity was not improved in Mo mice. Moreover, the overexpression (by two- to fourfold) of the cytokine tumour necrosis factor α (TNF α) in white adipose tissue, which may have a determinant role in the insulin resistance of the O mice, was unaffected by Mo. Likewise, overexpression of the *ob* gene in white adipose tissue was unchanged by Mo. In conclusion, Mo markedly improved glucose homeostasis in the *ob/ob* mice by an insulin-like action which appeared to be exerted distal to the insulin receptor tyrosine kinase step. The blood glucose-lowering effect of Mo was unrelated to overexpression of the TNF α and *ob* genes in O mice, but resulted at least in part from attenuation of liver insulin resistance by the reversal of pre-translational regulatory defects in these mice.

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Introduction

Considerable interest is currently paid to drugs that could alleviate insulin resistance. Insulin-sensitizers such as thiazolidinediones (reviewed in Saltiel & Olefsky 1996) and insulin-mimickers such as vanadium salts (reviewed in Brichard & Henquin 1995) are particularly promising; they open new therapeutic perspectives for the management of type 2 (non-insulin-dependent) diabetes.

Besides vanadium, other trace elements have been shown to exhibit insulin-like properties (Rossetti *et al.* 1990, McNeill *et al.* 1991, Brichard & Henquin 1995, Ozcelikay *et al.* 1996). Evidence has recently been presented that molybdenum could affect glucose metabolism *in vitro*. Addition of molybdate (Mo) to the incubation medium of isolated hepatocytes stimulated glycolysis and

accelerated glycogen degradation (Fillat *et al.* 1992). Mo also increased insulin receptor autophosphorylation and phosphorylation of its substrate (pp160) (Mooney & Bordwell 1992), and augmented glucose transport, oxidation and lipogenesis in adipocytes (Goto *et al.* 1992, Li *et al.* 1995a).

In vivo, oral Mo has little effect on plasma glucose concentrations in normal rats (D J Becker, AM Pottier and SM Brichard, unpublished data), but can markedly decrease blood glucose levels in rats made insulin-deficient and diabetic by injection of streptozotocin (Li *et al.* 1995b, Ozcelikay *et al.* 1996). The potential beneficial effects of Mo have not been explored in an animal model of type 2 diabetes.

In this study, we administered oral Mo to genetically obese, hyperinsulinaemic and insulin-resistant

ob/ob mice for 7 weeks, and evaluated parameters of glucose homeostasis and the expression of genes coding for key enzymes involved in hepatic glucose metabolism (glycolysis/gluconeogenesis). We also investigated whether Mo modified the expression of two genes in white adipose tissue that have an important role in the obese insulin-resistant syndrome: *ob* and tumour necrosis factor α (TNF α).

Materials and Methods

Animals

Obese hyperglycaemic (*ob/ob*) (male and female; 9–15 weeks old; 84 ± 2 g) and lean homozygous (+/+) (male only; 11–15 weeks old; 44 ± 2 g) mice were obtained from the colony of Aston University (Birmingham, UK). The sex of obese mice did not influence the severity of their diabetes. The animals were housed in individual cages at a constant temperature (22 °C) with a fixed 12 h light:12 h darkness cycle (lights on 0700–1900 h). Standard laboratory chow in powdered form (A03, Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France) composed of (wet weight): 50% carbohydrate, 5% fat, 23% protein and 22% water–minerals–cellulose was available *ad libitum* to all mice.

Experimental design

The mice were divided into three experimental groups: lean mice (L; $n=14$); untreated obese mice (O; $n=16$); obese mice treated with molybdate (Mo; $n=13$). In preliminary experiments performed in normal (lean) Wistar rats, we found no difference in basal and stimulated (after an oral glucose test) glycaemia between treated and untreated animals (D J Becker, unpublished data); a group of lean mice treated with Mo was therefore not included in the present study. At 0 week, the *ob/ob* mice (O and Mo groups) were matched for initial body weight (O: 81 ± 2 g; Mo: 82 ± 3 g) and fed plasma glucose levels (O: 15.6 ± 1.8 mM; Mo: 14.3 ± 1.5 mM). They were also matched, as seen *a posteriori*, for fed plasma insulin concentrations (O: 10.5 ± 1.7 nM; Mo: 12.8 ± 2.6 nM). To partially overcome an initial aversion to molybdate and ensure long-term fluid and food intake, the treated group received increasing amounts of sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, Merck, Darmstadt, Germany) in food and in two different drinking solutions consisting of either distilled water empirically supplemented with 85 mM NaCl or water slightly flavoured with cocoa. The solutions were freshly prepared every 3rd day. The initial concentration of sodium molybdate (0.2 g/l in solutions and 0.375 g/kg in food) was progressively increased to 1.5 g/l and 3.0 g/kg in drinking fluid and food respectively after 4 weeks, and then remained unchanged until the end of

the study (Fig. 1). The progressive increase in Mo concentrations and the maximal dose reached after 4 weeks (mg/kg body weight) were based on our previous studies in insulinopenic animals (A T Ozcelikay, unpublished data, Ozcelikay *et al.* 1996). The drinking solutions of O mice contained the same amount of NaCl or cocoa as the treated solutions.

Sampling and tests

On several occasions, tail vein blood was collected from fed mice (between 0830 and 0900 h) for determination of plasma glucose and insulin levels. All mice underwent an oral glucose tolerance test (OGTT) after 5 weeks of treatment. On the day before the test, food was removed at 1800 h. The test started at 0830 h. For the OGTT, glucose (40% in water) was introduced directly into the stomach through a fine gastric catheter at a dose of 2 g/kg body weight. Mice were gently wrapped in a towel to restrain them during injections or blood samplings.

After 7 (i.e. between 7 and 8) weeks of treatment, the mice were killed by decapitation between 0130 h and 0430 h (i.e. in the absorptive state). Blood samples were saved for determination of plasma glucose, insulin and non-esterified fatty acids (NEFA). Pancreas, liver and pairs of epididymal and inguinal fat pads were immediately removed, frozen in liquid nitrogen and stored at -70 °C for insulin or RNA extraction and enzyme measurements.

RNA extraction and Northern blot analysis

Total RNA was isolated with an acid guanidinium–thiocyanate–phenol–chloroform mixture after removal of liver glycogen as described previously (Ozcelikay *et al.* 1996). The concentration of RNA was determined by absorbance at 260 nm. At 260/280 nm, all samples had an absorbance ratio of about 1.8. For Northern blot analysis, RNA (15 μ g for white adipose tissue, 30 μ g for liver) was denatured in a solution containing 2.2 mM formaldehyde and 50% formamide (v/v) by heating at 95 °C for 2 min. RNA was then size-fractionated by 1% agarose gel electrophoresis, transferred to a Hybond-N membrane (Amersham International, Amersham, Bucks, UK) and cross-linked by ultraviolet irradiation. The integrity and relative amounts of RNA were assessed by methylene blue staining of the blot.

The cDNA probes were kindly supplied by Dr P Iynedjian for glucokinase (GK) (Iynedjian *et al.* 1987), Dr A Kahn for pyruvate kinase (L-PK) (Simon *et al.* 1983), Dr R W Hanson for phosphoenolpyruvate carboxykinase (PEPCK) (Yoo-Warren *et al.* 1983), Dr B Thorens for glucose transporter isoform (GLUT) 2 (Thorens *et al.* 1988), Dr W Fiers for TNF α (Fransen *et al.* 1985) and Dr D E James for GLUT4 (James *et al.* 1989). The *ob* cDNA probe was prepared as described previously (Becker *et al.* 1995). Probes were labelled with ^{32}P using the

Multiprime labelling system kit (Amersham). Hybridizations with GK, L-PK, PEPCK, GLUT2, GLUT4 or *ob* probes and subsequent washings of the membranes were performed as reported earlier (Becker *et al.* 1995, Ozcelikay *et al.* 1996). Hybridization with the TNF α probe was similar to that of PEPCK. The filters were thereafter exposed to Kodak X-OMAT AR films for 2–48 h at -70°C with intensifying screens. The same filters were successively hybridized with the different radiolabelled cDNA probes. Intensity of the mRNA bands on the blots was quantified by scanning densitometry (Ultrosan XL, LKB, Sweden). To verify that equivalent amounts of total RNA were loaded on each lane, blots were hybridized with a synthetic oligonucleotide specific for ribosomal 18S RNA (Chan *et al.* 1984).

Measurements of enzyme activities

The activities of GK (EC 2.7.1.1), L-PK (EC 2.7.1.40) and PEPCK (EC 4.1.1.32) were measured at 37°C , as described previously (Brichard *et al.* 1993, Ozcelikay *et al.* 1996). Results were expressed as milliunits (mU) enzyme per mg protein of liver cytosolic fraction, 1 mU enzyme being defined as that amount which catalyses the conversion of 1 nmol substrate per min under the assay conditions.

Preparation of insulin receptors, binding studies and tyrosine kinase activity assay

Insulin receptors were solubilized and partially purified with wheat germ-agglutinin-agarose beads (Le Marchand-Brustel *et al.* 1985, Heydrick *et al.* 1993) from homogenates of 350 mg liver tissue. Yields of glycoprotein were similar in the three groups of mice. Aliquots of receptor preparations (6 μg protein) were incubated in Hepes–NaCl buffer and BSA (0.1%) containing ^{125}I -labelled insulin (Amersham) in the absence and presence of increasing concentrations of unlabelled insulin. After 2 h at 20°C , insulin–receptor complexes were separated from free insulin by precipitation with polyethylene glycol (Le Marchand-Brustel *et al.* 1985). Non-specific binding (<5%) was defined as the radioactivity precipitated in the presence of $1\text{ }\mu\text{M}$ unlabelled insulin. The dissociation constant (K_d ; reciprocal of the affinity) and the insulin-binding capacity (receptor number) were calculated by regression analysis of the Scatchard plot.

Phosphorylation of poly(Glu 4 Tyr 1) by the insulin receptor kinase was measured as described previously (Le Marchand-Brustel *et al.* 1985). Partially purified insulin receptors were incubated for 1 h at 20°C in HEPES–NaCl buffer and BSA (0.2%) with and without 10^{-7} M insulin. Poly(Glu 4 Tyr 1) (final concentration 0.25 mg/ml) was then added for an additional 30 min before initiation of the phosphorylation reaction by addition of the ATP mixture (20 μM [$\gamma^{32}\text{-P}$]ATP, 2.5 μCi , 4 mM MnCl_2 ,

8 mM MgCl_2 , final concentrations). After 20 min, the reaction was stopped by applying samples to Whatman ET 31 filters and soaking the papers in 10% (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate. Radioactivity incorporated into the substrate was quantified by Cerenkov counting.

Analytical procedures

Plasma glucose was measured by a glucose oxidase method (Glucose Analyzer, Beckman, Fullerton, CA, USA). Plasma insulin was determined by a double-antibody radioimmunoassay, using rat insulin as standard (Novo Research Institute, Bagsvaerd, Denmark). Plasma NEFAs were assayed by a spectrophotometric method using a commercial kit (Wako Chemicals GmbH, Neuss, Germany). Pancreatic insulin was extracted by homogenization and sonication of the tissue in acidified ethanol. Liver glycogen was measured after extraction with KOH, precipitation in ethanol and hydrolysis with α -amylase-glucosidase.

Statistical analysis

Results are presented as means $\pm$ s.e. for the indicated numbers of mice. Comparisons between lean, untreated *ob/ob* and Mo-treated *ob/ob* mice were carried out by analysis of variance followed by the Newman–Keuls test for multiple comparisons. Differences were considered statistically significant at $P<0.05$.

Results

Body weights of the three groups of mice remained stable throughout the study. O mice were twice as heavy as L mice, and their body weight was unaffected by Mo treatment (Fig. 1).

Average fed plasma glucose levels of O mice were typically about 15–20 mM, being two- to threefold higher than those of L mice. Mo administration induced a concentration-dependent decrease in glycaemia. After 2 weeks of treatment, plasma glucose levels of Mo mice were already significantly lower than those of O mice ($P<0.05$) and decreased to values similar to those in L mice from the 4th week onward (Fig. 1) when the maximal amounts of Mo were supplied in the food and drinking fluid. Daily food intake was ~ 2.6 -fold higher in O than in L mice (8.8 ± 0.9 compared with 3.4 ± 0.1 g/day; $P<0.001$) and was not significantly reduced in Mo mice (7.6 ± 0.5), whereas the polydipsia of O mice (15.3 ± 2.4 ml/day compared with 5.2 ± 0.4 ml/day in L mice; $P<0.001$) was significantly attenuated by the treatment (to 9.1 ± 0.5 ml/day; $P<0.01$). Thus, at week 4, daily consumption of molybdenum element per mouse averaged 174 ± 9 mg/kg body weight.

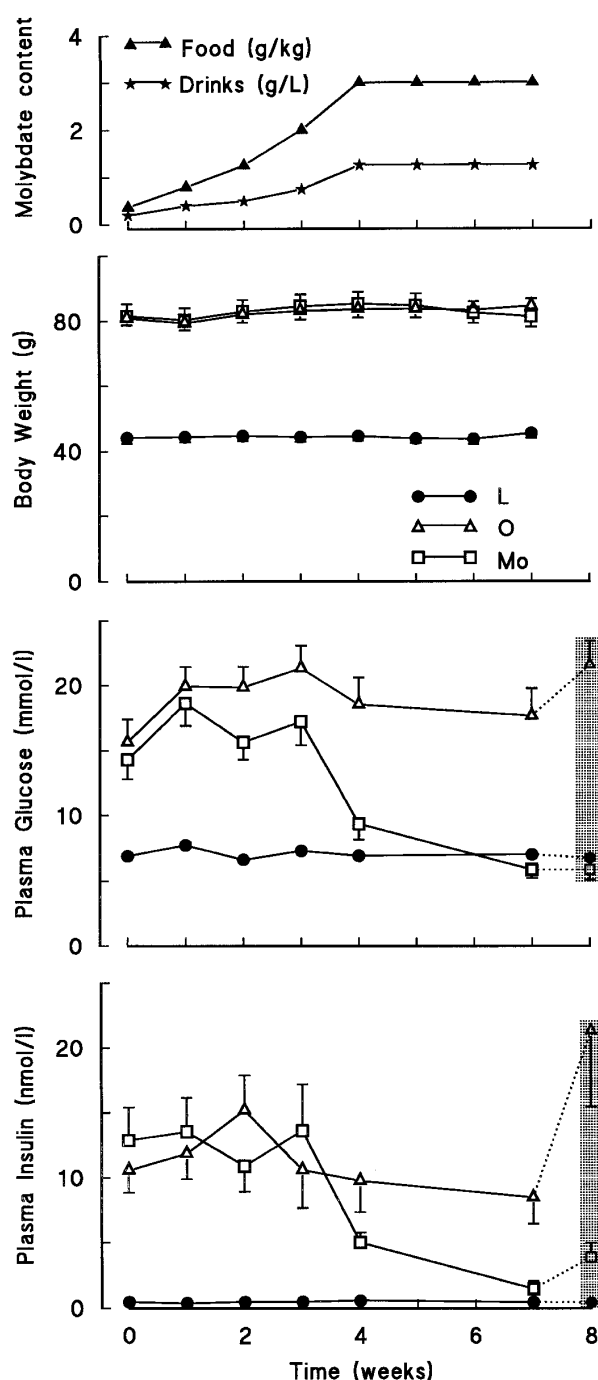


Figure 1 Effects of molybdate treatment on body weight, plasma glucose and insulin levels of the *ob/ob* mice. Three groups were studied: untreated lean (L) mice, untreated *ob/ob* (O) mice, and *ob/ob* mice treated with molybdate (Mo). Molybdate was given as increasing amounts of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ in drinking solutions and in food. All measurements were made between 0830 and 0900 h except those in the stippled area (0200–0400 h). Values are the mean $\pm$ S.E.M. for 14, 16 and 13 mice in the respective groups.

Average fed plasma insulin levels of O mice (11.1 ± 0.9 nM) were ~ 25 -fold higher than those of L mice (0.5 ± 0.1 nM). The decrease in insulinaemia induced by Mo was concomitant with the decrease in glycaemia, and the concentrations reached at the end of the study were not different from those in L mice (Fig. 1). Mo-induced changes in glycaemia and insulinaemia were also observed when the animals were in an absorptive state (measurements at 0200–0400 h; stippled area in Fig. 1).

As reported previously (Lombardo *et al.* 1983), plasma NEFA levels were similar in O and L mice (0.19 ± 0.01 g/l and 0.21 ± 0.01 g/l respectively; $n=14$ –16; values obtained at the end of the study). They were unchanged by administration of Mo (0.20 ± 0.02 g/l; $n=13$).

After an overnight fast (time zero of the OGTT), plasma glucose levels of Mo mice were lower than those of O mice (7.1 ± 0.5 compared with 12.6 ± 1.7 mM, $P<0.01$), and were approaching the values in L mice (4.8 ± 0.3 mM) (Fig. 2). Fasting plasma insulin levels of Mo mice were reduced by $\sim 50\%$ compared with those of O mice (0.7 ± 0.2 compared with 1.5 ± 0.3 nM; $P<0.01$), but remained higher than those of L mice (0.06 ± 0.01 nM; $P<0.05$) (Fig. 2).

During the OGTT, plasma glucose levels remained less than 15 mM in L mice, but increased to more than 30 mM and remained increased throughout the test in O mice (Fig. 2). In Mo mice, glucose concentrations were consistently lower ($P<0.05$ or less) than in O mice and decreased to similar values as in L mice at 120 min. Total glucose area (above zero level) in Mo mice was 33% smaller than in O mice ($P<0.01$), but remained greater than that in L mice (Fig. 2). Plasma insulin levels of L mice were less than 0.2 nM, and a significant ~ 2.2 -fold increase was observed between 0 min and each time point of the OGTT ($P<0.001$ or less by paired t-test; scarcely visible in Fig. 2). Insulinaemia of O mice was markedly increased at all time points (8- to 24-fold), but did not increase further in response to glucose. Hyperinsulinaemia was consistently reduced by Mo treatment ($P<0.05$ or less compared with O mice), although the response at 30 min was not significantly restored. Total insulin area was 11-fold higher in O than in L mice and was reduced by $\sim 50\%$ after administration of Mo.

Pancreatic insulin reserves (nmol/pancreas) were similar in L and O mice (19.4 ± 1.2 and 15.4 ± 3.6 respectively) and about twofold higher in Mo-treated animals (41.3 ± 4.7 nmol/pancreas; $P<0.01$).

Hepatic glycogen stores were not modified in the O mice (56.7 ± 2.2 mg/g liver compared with 56.1 ± 2.3 mg/g liver in L mice), but the expression of certain genes involved in key steps of glucose metabolism was altered. The abundance of GK mRNA was not modified in the liver of O mice ($\sim 71\%$ of control L mice), whereas that of L-PK mRNA was markedly increased ($\sim 350\%$ of L mice) (Fig. 3). Accordingly, the activities of GK and

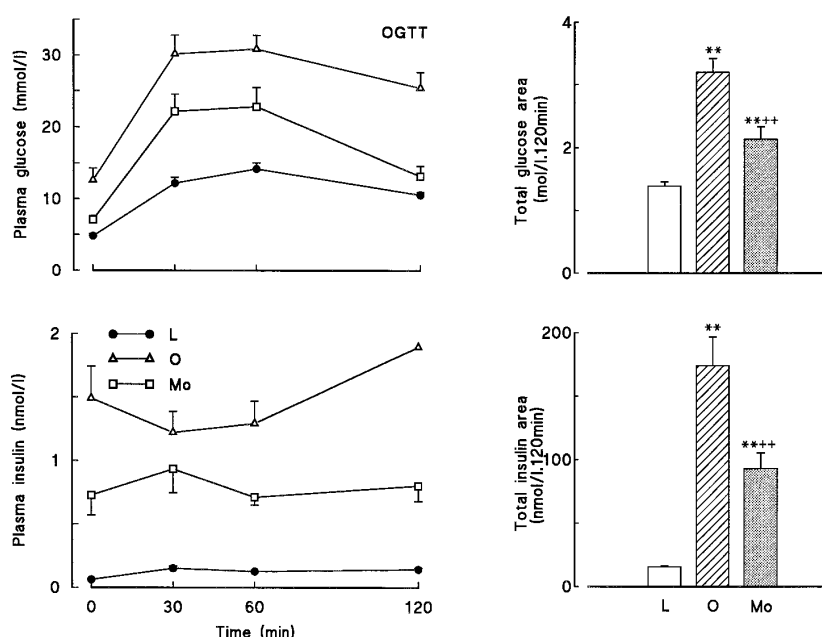


Figure 2 Effects of molybdate on tolerance to oral glucose (OGTT) in *ob/ob* mice. *Left panels:* plasma glucose and insulin levels during OGTT in lean (L), untreated *ob/ob* (O) and molybdate-treated (Mo) *ob/ob* mice. *Right panels:* total glucose and insulin areas (above zero levels) during the OGTT. The test was performed after 5 weeks of treatment. Values are the means $\pm$ S.E.M. for 14, 16 and 13 mice. ** $P < 0.01$ compared with L mice; +++ $P < 0.01$ compared with O mice.

L-PK were unchanged or augmented in O mice (Fig. 3). Mo treatment did not influence hepatic glycogen (54.0 ± 3.3 mg/g), increased GK mRNA and activity (by $\sim 120\%$ and 61% compared with O values), and had no, or only a mild, effect on the already increased levels of L-PK mRNA and activity (Fig. 3). mRNA levels and activity of the gluconeogenic enzyme, PEPCK, were increased in the liver of O mice (sixfold and 57% respectively), but were corrected by Mo treatment (Fig. 3). GLUT2 mRNA concentrations were augmented 4.7-fold in O mice, but were unaltered by treatment with Mo (Fig. 3). Hepatic 18S rRNA levels were similar in the three groups of mice (data not shown).

Partially purified insulin receptors were prepared from liver of the three groups of mice. As described previously (Kahn *et al.* 1973), insulin binding was decreased by $\sim 30\%$ in O mice compared with L mice. At variance with another study in *ob/ob* mice from the same colony (Lord *et al.* 1983), Scatchard analysis of the present data indicated that this decrease was due mainly to a reduction in binding affinity (K_d 0.30 ± 0.05 nM in O mice and 0.16 ± 0.02 nM in L mice; $n=11$ for each group; $P < 0.05$), rather than to a change in apparent receptor number (total binding capacity 1.12 ± 0.11 fmol/ μ g protein in O mice and 0.99 ± 0.10 fmol/ μ g protein in L mice) (Fig. 4). Differences in diet, period of fasting and the use of partially

purified receptors could explain this discrepancy. Mo treatment restored insulin binding to values obtained in L mice, perhaps through the attenuation of hyperinsulinaemia. This restoration was accounted for by an increase in receptor number (1.61 ± 0.28 ; $P < 0.05$ compared with L mice), with no change in impaired affinity (K_d 0.25 ± 0.04 nM) (Fig. 4). Insulin receptor tyrosine kinase activity was measured towards the copolymer poly(Glu⁴Tyr¹), and expressed as pmol ³²P incorporated per min per mg of glycoprotein present in the receptor preparations. Basal tyrosine kinase activity measured in the absence of insulin was similar in all groups (L: 49 ± 4 , O: 48 ± 4 , Mo: 54 ± 5). Likewise, there was no difference in stimulated tyrosine kinase activity, measured in the presence of 10^{-7} M insulin, between the groups (L: 68 ± 5 , O: 68 ± 8 , Mo: 70 ± 7).

We next examined whether Mo treatment of obese mice modified the abnormal expression of genes which are believed to have a determinant role in the pathogenesis of the obese insulin-resistant syndrome. Expression of the *ob* gene was increased by 44% and 113% in epididymal and inguinal white adipose tissue of O mice, and was unaffected by Mo treatment (Fig. 5). The cytokine TNF α was also overexpressed in both adipose tissue sites (four- and twofold) and remained unchanged in Mo mice (Fig. 5). GLUT4 mRNA was decreased in epididymal adipose

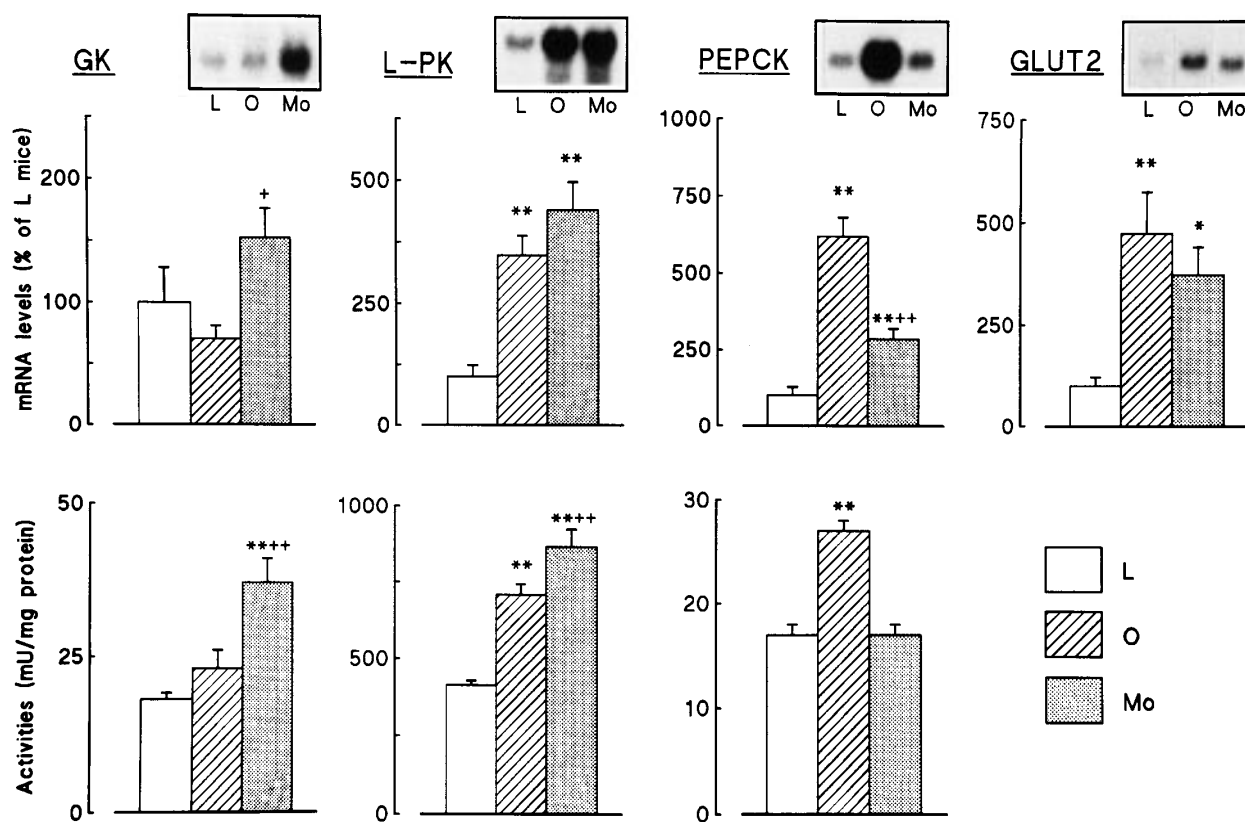


Figure 3 Effects of molybdate treatment on mRNA levels and activities of hepatic glucokinase (GK), L-type pyruvate kinase (L-PK), phosphoenolpyruvate carboxykinase (PEPCK), and GLUT2 transporter mRNA in lean (L), untreated *ob/ob* (O) and molybdate-treated *ob/ob* (Mo) mice. Values are the means $\pm$ S.E.M. for five to eight mice (mRNAs) and 13–16 mice (activities). mRNA levels were quantified by scanning densitometry of autoradiographic signals obtained from Northern blots as shown in the inserts, and the results were expressed as percentages of values in L mice. L-PK activity was measured at 4 mM phosphoenolpyruvate (maximal activity of the enzyme). * $P < 0.05$, ** $P < 0.01$ compared with L mice; + $P < 0.05$, ++ $P < 0.01$ compared with O mice.

tissue of O mice, and was again unmodified by Mo administration. There was no significant difference in GLUT4 mRNA in inguinal fat pads of the three groups (Fig. 5).

Discussion

The present study has shown that molybdate is an effective antihyperglycaemic agent in obese-diabetic animals with severe insulin resistance. The blood glucose-lowering action of Mo was marked and sustained, and observed both during an oral glucose load and under basal conditions. It was associated with a substantial reduction of hyperinsulinaemia and an apparent increase in pancreatic insulin stores. The glucose-lowering effect of Mo may be partly related to attenuation of hepatic glucose production (and possibly also to increased glucose usage), but appears to be unrelated to the overexpression of the TNF α and *ob* genes.

Mutation of *ob* leads to undetectable plasma levels of *ob* protein (leptin) (Halaas *et al.* 1995) and increased mRNA

concentrations – as part of a feedback loop – (Zhang *et al.* 1994), and results in profound obesity with a non-insulin-dependent form of diabetes. Injection of leptin to *ob/ob* mice reduces *ob* mRNA (Pellemounter *et al.* 1995), and produces dose-dependent weight loss and decreases in serum insulin and glucose levels (Halaas *et al.* 1995, Slieker *et al.* 1996). Leptin-induced improvement of diabetes appears to result in part from intrinsic effects of the hormone on glucose metabolism, but also from weight loss (Schwartz *et al.* 1996). Whatever the exact mechanisms, those involved in Mo action appear to be totally different, as treatment with the element improves glucose homeostasis independently from changes in body weight and *ob* expression.

Recent data suggest that TNF α also has a key role in the pathogenesis of the obese insulin-resistant syndrome. TNF α expression is increased in the adipose tissue in human type 2 diabetes and in many experimental models of obesity (including the *ob/ob* mice) (Hotamisligil & Spiegelman 1994). In one of these models, neutralization of TNF α improved insulin sensitivity by increasing

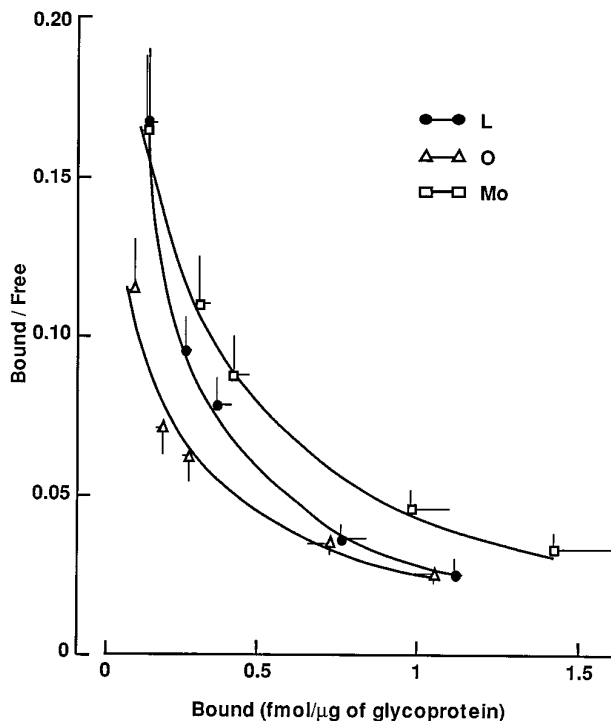


Figure 4 Scatchard plots of insulin binding to receptors partially purified from liver of lean (L), untreated *ob/ob* (O) and molybdate-treated *ob/ob* (Mo) mice. Specific binding was normalized to protein concentration in the wheat germ eluate. Values are the means $\pm$ S.E.M. for 11 different mice per group.

insulin-stimulated tyrosine phosphorylation on the insulin receptor and on its substrate (Hotamisligil & Spiegelman 1994). However, as Mo did not modify the expression of TNF α in adipocytes, its beneficial effects are unlikely to be related to reduced TNF α production. In this respect, the action of Mo differs from that of pioglitazone, a drug from the thiazolidinedione class of insulin-sensitizers (Hofmann *et al.* 1994).

Several other mechanisms could, theoretically, contribute to the improvement of glucose homeostasis induced by Mo. An inhibition of intestinal glucose reabsorption is unlikely to be the sole explanation because the action of Mo was detectable in fasted and in fed rats. A reduction in NEFA levels, with subsequent attenuation of the glucose-fatty acid cycle (Randle *et al.* 1963), may also be excluded, because plasma NEFA concentrations were unaffected by the treatment. An insulin-like action similar to that described *in vitro* may be of major importance in the *in vivo* effects of Mo. This is supported by the significant decrease in blood glucose levels coupled with a marked lowering of the hyperinsulinaemia, and by the significant effects on hepatic glucoregulatory enzymes described below. Our *in vivo* observations, however, contradict the report that Mo stimulates glycogen degradation in isolated hepatocytes (Fillat *et al.* 1992).

In the liver of *ob/ob* mice, the activities of the key glycolytic enzyme L-PK, and the gluconeogenic enzyme PEPCK were increased. This is in keeping with the acceleration of both the glycolytic and gluconeogenic pathways found in most (Seidman *et al.* 1967, Kreutner *et al.* 1975, Hue & van de Werve 1982), but not all (Ferber *et al.* 1994), reports on the liver of these mice. In the present study, evidence that these abnormalities were mediated at the pre-translational level is further presented by the increased mRNA levels. As GK mRNA was unchanged and PEPCK mRNA augmented in spite of hyperinsulinaemia, the insulin-induced regulation of these two genes, but not of L-PK, appears to be impaired in *ob/ob* mice. This is in agreement with a recent observation in another animal model of type 2 diabetes (Noguchi *et al.* 1993). Mo treatment decreased the hyperinsulinaemia of *ob/ob* mice, increased GK mRNA and decreased PEPCK mRNA, thereby leading to a possible enhancement of hepatic glucose usage and to inhibition of glucose overproduction. This suggests a reversal of liver insulin resistance at the pre-translational level.

We next determined whether the action of Mo on the liver was mediated by insulin receptor or post-receptor events. Insulin binding was decreased in O mice, as reported previously (Kahn *et al.* 1973), and was restored by Mo treatment. In spite of this correction, overall tyrosine kinase activity was not improved. As far as *in vitro* assays may reflect *in vivo* changes, these data taken together suggest that the improvement of glucose homeostasis brought about by Mo may occur distal to the insulin receptor tyrosine kinase step. Similar conclusions were reached for the effects of vanadate in *ob/ob* mice (Meyerovitch *et al.* 1991).

GLUT4 and hexokinase II mRNA were unaltered in heart and hind-limb skeletal muscle of O mice and unchanged by Mo treatment (B Reul, unpublished data). As other factors have a determinant role in muscle insulin resistance (such as functional defects of GLUT4) (Kahn 1992), the possibility that the antidiabetic properties of Mo can also be ascribed to an insulin-like action on this tissue cannot be ruled out. By contrast, GLUT4 mRNA levels were decreased in epididymal white adipose tissue of O mice (Braithwaite *et al.* 1995). These low levels were not corrected by Mo treatment. Thus there was no conclusive evidence for an insulin-like effect of Mo in adipose tissue *in vivo*. Similarly, the altered expression of TNF α was unmodified. This absence of effects in white adipose tissue is in line with the lack of change in adipose tissue metabolism of streptozotocin-diabetic rats treated with molybdate or of *ob/ob* mice treated with vanadate (Brichard *et al.* 1990, Ozcelikay *et al.* 1996).

An effect of Mo on pancreatic β -cells must also be considered. The β -cell population of obese mice is initially greater than that in lean mice, but declines markedly with advancing age and diabetes (Bailey *et al.* 1982). At the end of the study, the pancreatic insulin content was twofold

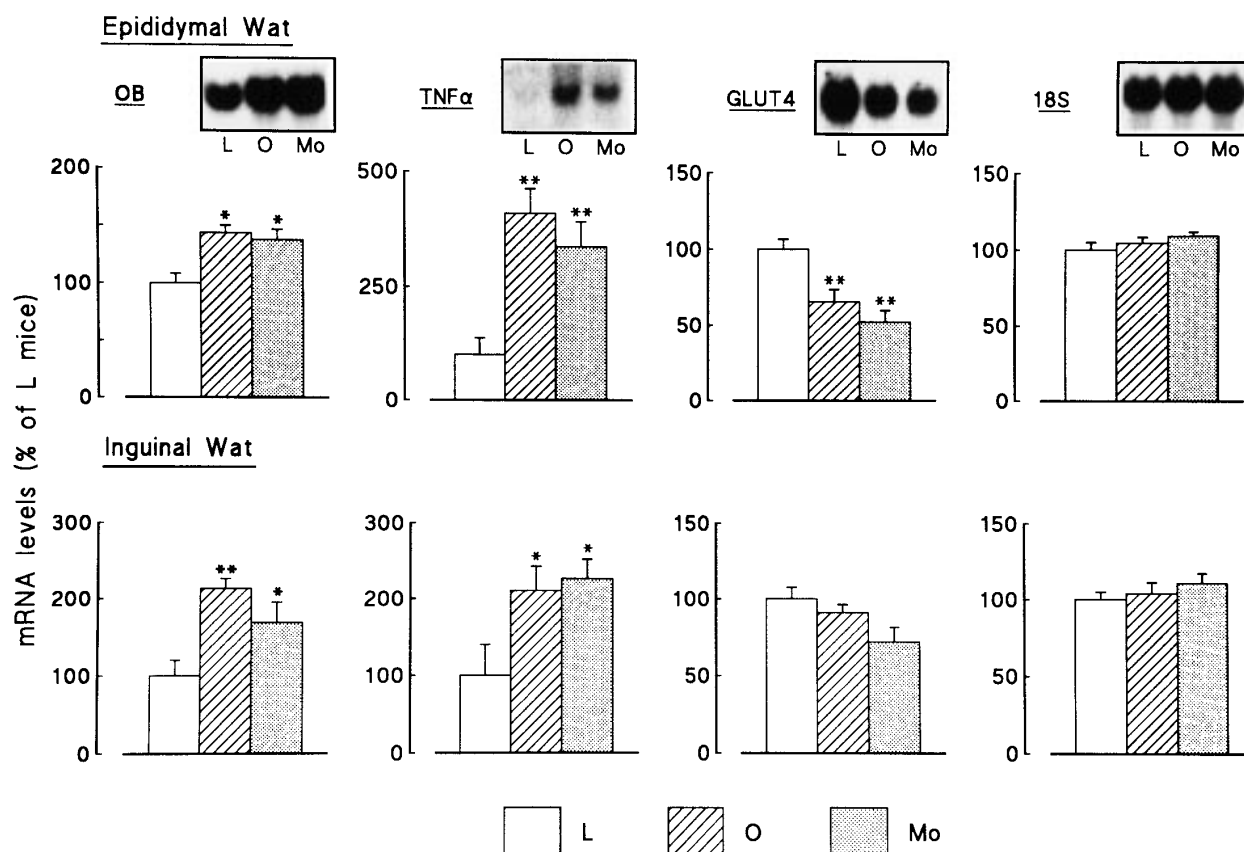


Figure 5 Influence of molybdate treatment on *ob*, *TNFα* and *GLUT4* mRNA, and 18S rRNA levels in epididymal and inguinal white adipose tissue (Wat) of *ob/ob* mice. Values are the means $\pm$ S.E.M. for five to eight lean (L), untreated *ob/ob* (O) mice and molybdate-treated *ob/ob* (Mo) mice. mRNA and rRNA levels were quantified by scanning densitometry of autoradiographic signals obtained from Northern blots as shown in the inserts. The results are expressed as percentages of values in L mice. * $P < 0.05$, ** $P < 0.01$ compared with L mice.

higher in ~5-month-old obese mice treated with Mo than in untreated O mice. We interpret this higher content as a sparing effect by the element. Indeed, the attenuation of insulin resistance by Mo may decrease the needs for endogenous insulin and preserve the stores of the hormone.

Although the effects of Mo on glucose metabolism are reminiscent of those produced by vanadate in *ob/ob* and *db/db* mice (Brichard *et al.* 1990, Meyerovitch *et al.* 1991), they may not be identical. First, vanadate and molybdate inhibit different phosphotyrosine phosphatases, ultimately to activate a similar non-insulin-dependent cytosolic tyrosine kinase, which is believed to mediate their effects (Li *et al.* 1995a). Hence, the trigger of the insulin-like cascade would be different. Secondly, the insulin-like properties of vanadate and molybdate have been found to be synergistic *in vitro* (Mooney & Bordwell 1992). If this should be confirmed *in vivo*, it would carry significant therapeutic potential.

There were no toxic side-effects of Mo on renal or hepatic function in streptozotocin-diabetic rats chronically treated with the element (Ozelikay *et al.* 1996). Recent clinical studies with vanadium compounds have also been encouraging. Two or three weeks of treatment of human type 2 diabetic patients with small doses of these agents improved the inhibition of hepatic glucose production by insulin and the stimulation of peripheral glucose disposal (Cohen *et al.* 1995, Goldfine *et al.* 1995). Whether Mo could be alternative or complementary to vanadium therapy remains speculative. Further animal studies are warranted.

In conclusion, Mo proved to be an effective blood glucose-lowering agent in severely diabetic and insulin-resistant mice by an insulin-like action which involved post-insulin receptor tyrosine kinase events. Its anti-hyperglycaemic effects were unrelated to changes in *TNFα* and *ob* overexpression, but resulted partly from attenuation of liver insulin resistance subsequent to reversal of altered pre-translational regulatory mechanisms.

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