ORIGINAL ARTICLES

Comparative study on the preventing effects of oral vanadyl sulfate and dietary restriction on the age-related glucose intolerance in rats

Michela Novelli, Vincenzo De Tata, Vanna Fierabracci, Michele Barbera, Raffaele Rossetti, and Pellegrino Masiello

Department of Experimental Pathology, Medical Biotechnologies, Infectivology and Epidemiology, University of Pisa, Pisa, Italy

ABSTRACT. ***Background and aims:*** *Aging is associated with a progressive impairment of glucose tolerance. The aim of this study was to explore the protective effects of the chronic oral administration of the insulino-mimetic agent vanadyl sulfate ($VOSO_4$) as compared with those exerted by a long-lasting dietary restriction.* ***Methods:*** *Male Sprague-Dawley rats, either fed ad libitum (AL) or subjected to 40% dietary restriction (DR), were used. $VOSO_4$ (0.5 mg/mL drinking water) was administered to a subgroup of AL rats for two months, starting at 16 months of age. Rats were subjected to an intravenous glucose tolerance test (IVGTT) at 16 and 18 months of age. Finally, the beta-cell responsiveness to glucose was evaluated* in vitro *by the isolated perfused pancreas preparation.* ***Results:*** *The IVGTT performed in 16-month-old rats showed that DR prevented the development of the moderate glucose intolerance observed in AL rats. The IVGTT performed at 18 months of age confirmed the beneficial effect of DR and showed that $VOSO_4$ was able to prevent the further age-related progression of glucose intolerance observed in AL rats. Pancreas perfusion studies showed that no increase in insulin secretion occurred in both $VOSO_4$-treated and DR rats with respect to the age-matched AL controls, consistently with the* in vivo *observation of post-loading insulinaemic changes.* ***Conclusions:*** *On the basis of these results, we conclude that the beneficial effect of both treatments is mostly related to an improvement of tissue sensitivity to insulin rather than to an insulinotropic effect.*

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INTRODUCTION

A well-known feature of normal aging in both humans and experimental animals is the progressive impairment of glucose tolerance (1), which can represent a relevant risk factor for the development of type 2 diabetes mellitus (2). This age-related alteration of glucose homeostasis is usually considered to be dependent on either a decline in the insulin secretory capability of pancreatic islets with increasing age (3, 4) or an impairment of the normal sensitivity of peripheral tissues to insulin (5, 6), or both, although the mechanisms involved have not been fully clarified.

We have previously reported that a short-term oral administration of vanadyl sulfate, a well-known insulinomimetic agent, can rapidly normalize the imbalance of glucose metabolism observed in senescent (24-month-old) rats, probably by restoring the ability of peripheral tissues to utilize circulating insulin efficiently (7). The aim of this research was to explore whether a 2-month period of oral administration of low pharmacological doses of vanadyl sulfate could protect the animals against the occurrence or the aggravation of this age-related alteration in glucose homeostasis. We also wanted to directly compare the effect of $VOSO_4$ with that of long-lasting dietary restriction, an intervention whose anti-aging properties are well documented (8-10). The utilization of glucose tolerance tests *in vivo* coupled with evaluation of insulin secretion from the isolated perfused pancreas *in vitro* would also allow us to know whether the putative benefits of each intervention should be ascribed to an improvement of pancreatic insulin secretory capabilities or an amelioration of peripheral insulin sensitivity.

Key words: Aging, calorie restriction, glucose tolerance, insulin resistance, isolated perfused pancreas, rats.

Correspondence: P. Masiello, MD, Dipartimento di Patologia Sperimentale & B.M.I.E., Università di Pisa, Scuola Medica, Via Roma 55, 56126 Pisa, Italy.
E-mail: p.masiello@ipg.med.unipi.it

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METHODS

Animals and dietary restriction

Male Sprague-Dawley rats of 2 months of age purchased from Harlan Italy (Milan, Italy), were housed in the animal facility of our Department under artificial lighting (12-h light-dark cycle) and fed a standard laboratory chow (Harlan Teklad 2018/TRM, Harlan Italy). These rats were randomly divided into two groups: 1) *ad libitum* fed rats (AL, controls); 2) rats subjected to 40% food restriction (40% DR, i.e. rats received 60% of the amount of food consumed daily by the AL group). The experimental protocol followed the Principles of Laboratory Animal Care (US NIH publication No. 83-25, revised 1985) as well as the recommendations of the Italian law for the use of experimental animals (DL No. 116/1992) and was approved by the Ethical Committee of the University of Pisa Medical School.

Procedures

Vanadyl sulfate ($VOSO_4$) treatment. At 16 months of age, control AL rats were divided into two subgroups: 1a) controls receiving no treatment; 1b) vanadyl-treated rats, receiving tap water supplemented with 0.5 mg/ml vanadyl sulfate. The $VOSO_4$ solution was prepared freshly every other day.

Intravenous glucose tolerance test (IVGTT). Just before starting $VOSO_4$ administration (i.e. when rats were aged 16 months), non-fasting AL and 40% DR rats were subjected to an intravenous glucose tolerance test (0.75 g/kg b.w. as a 33% glucose solution). IVGTT was again performed at the age of 18 months, with a subgroup of AL rats being treated with $VOSO_4$ for two months. Blood samples were collected from the tail vein of conscious rats 0, 5, 15, 30 and 60 min after glucose administration. Plasma glucose was measured by the glucose-oxidase method using a commercially available kit (GLUCINET, Sclavo Diagnostics, Siena, Italy). Plasma insulin was measured according to Herbert et al. (11), using rat insulin as a standard, anti-insulin antibody from ICN (Milan, Italy) and labelled insulin from Linco (St. Charles, MO, USA).

Glucose tolerance was quantitated by two parameters: ΔG (integrated increase of glycaemia over baseline over a period of 60 min after the i.v. glucose load) and K coefficient (glucose disappearance rate after glucose injection calculated from the slope of the logarithm of the postload plasma glucose concentrations between the peak values at 5 min and the value at 30 min and expressed as percentage per minute). Insulin secretion during the IVGTT was quantitated as the incremental insulin values integrated over 60 min after the load (ΔI); the insulinogenic index ($\Delta I/\Delta G$) was also calculated.

Pancreas perfusion. At 18 months of age both AL (either treated or untreated with oral $VOSO_4$ for two months) and 40% DR rats were anesthetized with 100 mg/kg b.w. sodium pentobarbital intraperitoneally. The *in situ* isolated pancreas preparation was a modification of the method of Penhos et al. (12). Perfusate was a modified Krebs-Ringer bicarbonate buffer with 4% dextran T 40 and 0.25% bovine serum albumin, and was equilibrated with 95% O_2 and 5% CO_2. Flow rate was kept constant at 4 ml/min by a peristaltic pump for the entire perfusion period which lasted for about 70 min. The first 30 min served as an equilibration period and will not be shown on graphs. Effluent from the portal vein was collected over 1 min time intervals into tubes which were immediately frozen and stored at –20°C until insulin radioimmunoassay.

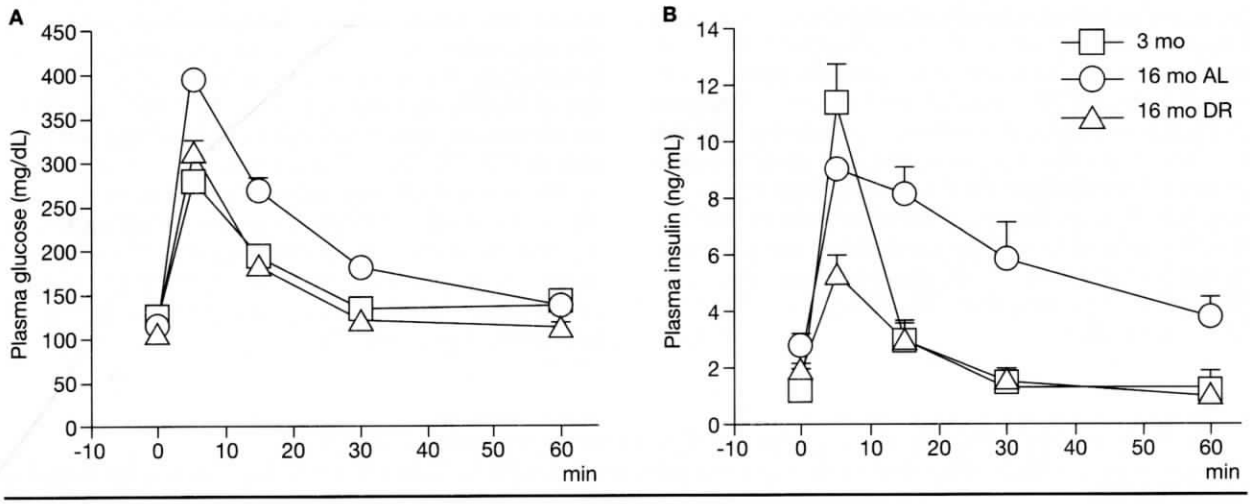

Fig. 1 - *Plasma glucose (A) and insulin (B) levels during an intravenous glucose tolerance test (0.75 g/kg b.w.) performed in 3-month-old (□), 16-month-old AL (○), and 16-month-old DR (Δ) male Sprague-Dawley rats. Data are means ± SEM of 6-8 observations for each experimental group. AL= ad libitum fed rats; DR= dietary restricted rats.*

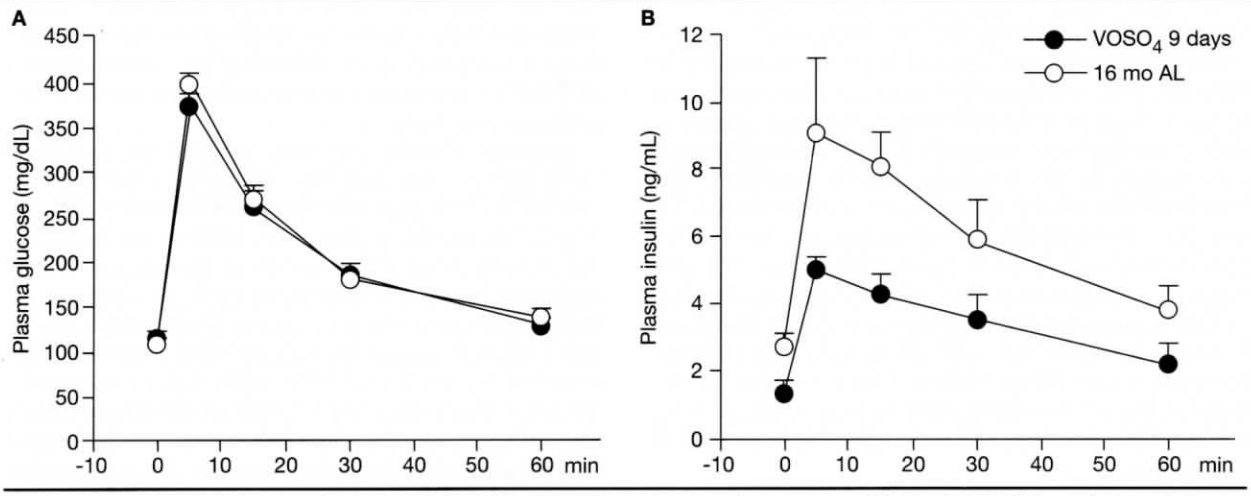

Fig. 2 - *Plasma glucose (A) and insulin (B) levels during an intravenous glucose tolerance test in 16-month-old ad libitum fed rats following 9 days of $VOSO_4$ treatment. Data are means ± SEM of 6 observations for each group.*

The perfusion protocol consisted of three consecutive periods of 15 min each, in the presence of 2.8 mM, 16.7 mM and 2.8 mM glucose, respectively. The insulin secretion was calculated from the insulin concentration in the perfusate (ng/ml) multiplied by flow rate.

Statistical analysis

Data are given as means ± SEM. Statistical significance was evaluated by factorial analysis of variance (ANOVA), followed by the two-tailed unpaired Student's *t*-test as a method of *post-hoc* analysis to assess two-by-two differences, when appropriate. A *p*-value of <0.05, at least, was considered as significant.

RESULTS

Figure 1 shows the results of an intravenous glucose tolerance test (IVGTT) performed in 16-month-old rats, either fed *ad libitum* (AL) or subjected to a regimen of dietary restriction (40% DR) starting at 2 months of age. For comparison, in the same figure, an IVGTT performed in a group of 3-month-old rats of the same strain is also shown. In young rats, the intravenous injection of glucose (0.75 mg/kg b.w.) caused a rapid elevation in plasma glucose and insulin levels, which both peaked 5 min after the load and then rapidly decreased. In 16-month-old AL rats, plasma glucose levels also peaked 5 min after glucose injection, but the subsequent return to baseline required a

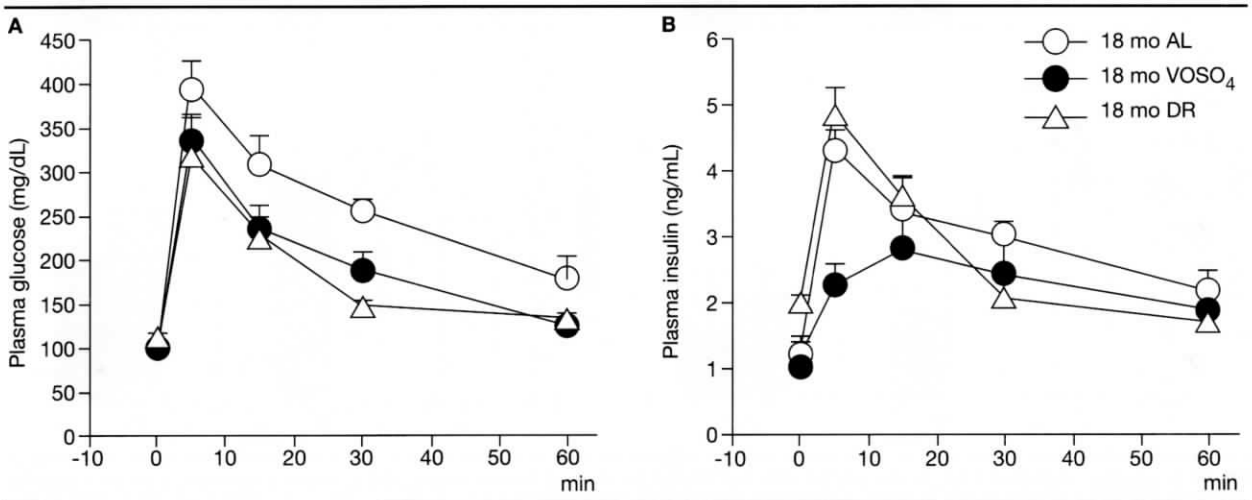

Fig. 3 - *Plasma glucose (A) and insulin (B) levels during an intravenous glucose tolerance test (0.75 g/kg b.w.) performed in 18-month-old untreated AL (○), $VOSO_4$-treated AL (●) and DR (Δ) male Sprague-Dawley rats. Data are means ± SEM of 4-6 observations for each group.*

longer time than in young rats. In such animals, a prolonged post-loading elevation of circulating insulin was correspondently observed. Interestingly, in 16-month-old 40% DR rats, post-loading glycaemic values were very similar to those observed in young animals and plasma insulin levels were also comparable, with the exception of the 5-min peak, which was significantly reduced. In 16-month-old AL rats, the quantitative analysis of the IVGTT (see Fig. 4) confirmed the significant elevation of the post-loading integrated increase of blood glucose (ΔG) with respect to young controls, as well as the preventing effect of DR despite reduced circulating insulin levels. However, it should be stressed that in AL animals the concomitantly increased plasma insulin levels were able to normalize the post-loading glucose disappearance rate, as indicated by a K coefficient not significantly different from that of younger rats.

Then, an oral $VOSO_4$ administration (0.5 mg/ml in the drinking water) was started in a subgroup of these AL rats and another IVGTT was performed after 9 days of treatment in order to check the early changes induced by the treatment. While no significant difference in the glycaemic profile was observed between $VOSO_4$-treated and untreated rats, plasma insulin levels were significantly ($p<0.05$) reduced in treated rats at any time after glucose injection (Fig. 2), suggesting that only a few days of $VOSO_4$ administration could induce an improvement of insulin sensitivity.

After two months of $VOSO_4$ treatment in a subgroup of AL rats (i.e. when animals were aged 18 months), a new IVGTT was performed in both AL and 40% DR rats (Fig. 3). By comparing the results obtained in 18-month-old rats with those of two months earlier (also see IVGTT analysis in Fig. 4), it is clear that in untreated AL rats a further impairment in glucose tolerance occurred between 16 and 18 months of age (as indicated by the significant decrease of K coefficient at 18 months), with a concomitant reduction of the insulinaemic response. $VOSO_4$ treatment appears to prevent the aggravation of glucose intolerance in aging animals; indeed the glycaemic curve of vanadyl-treated 18-month-old rats was similar to that observed in 16-month-old animals. Interestingly, this relatively preserved glucose tolerance was accompanied by circulating insulin levels significantly lower than those observed in untreated animals, thereby indicating an improvement of the peripheral sensitivity to insulin. This conclusion is also sup-

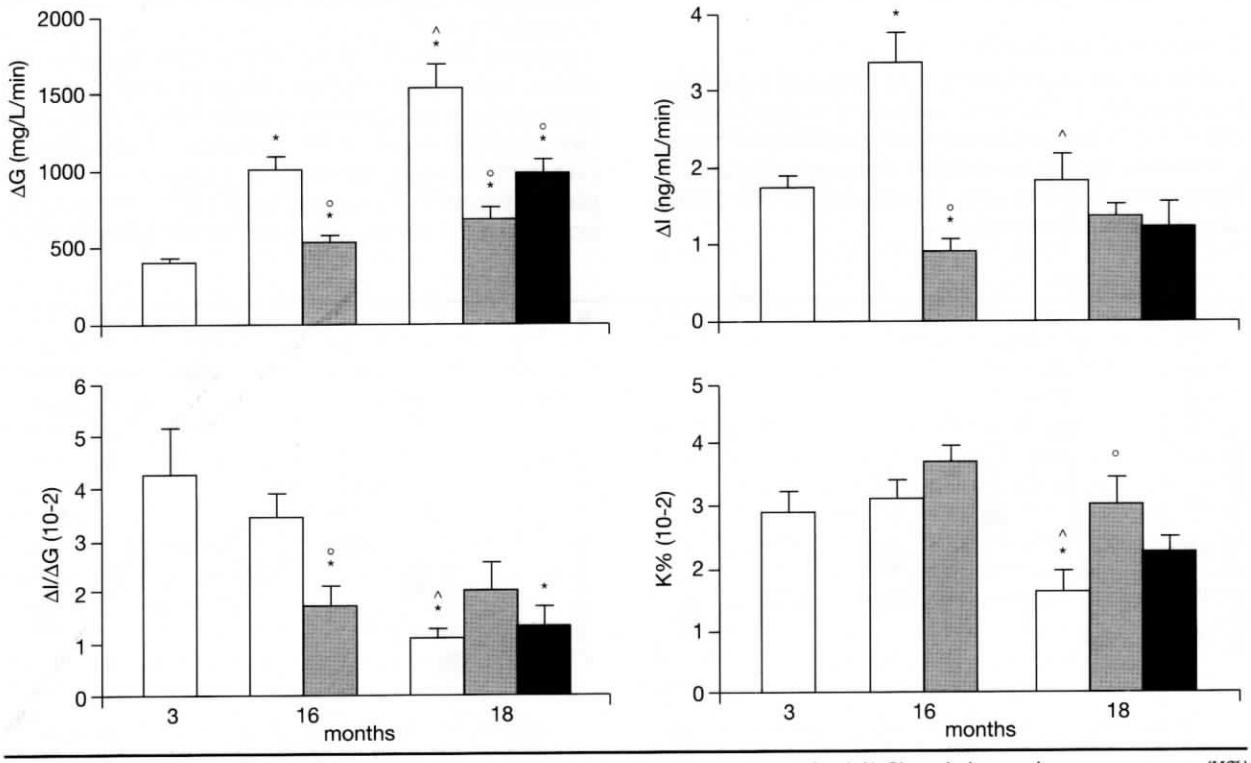

Fig. 4 - *Integrated increase in plasma glucose (ΔG) and insulin (ΔI) levels, insulinogenic index (ΔI/ΔG), and glucose disappearance rate (K%) during the IVGTTs of Figs. 1 and 3. Data are means ± SEM of 4-6 observations for each group. Statistical analysis (post-hoc Student's t test): *p<0.05, at least, vs 3-month-old rats; °p<0.05, at least, vs age-matched AL rats; ^p<0.05, at least, between 16- and 18-month-old AL rats. White columns: untreated AL rats; Grey columns: DR rats; Black columns: $VOSO_4$-treated rats.*

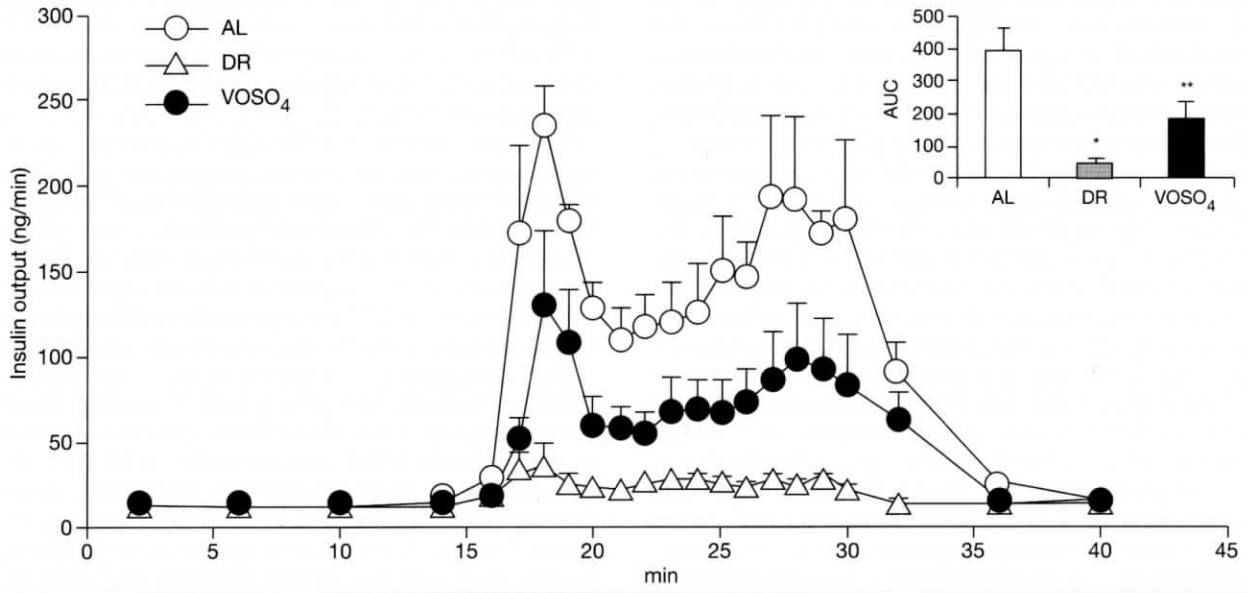

Fig. 5 - *Glucose (G)-stimulated insulin secretion from the isolated perfused pancreas of 18-month-old untreated AL (○), $VOSO_4$-treated AL (●) and DR (Δ) male Sprague-Dawley rats. Data are means ± SEM of 4-6 observations for each group. The insert shows the integrated insulin output in response to 16.7 mM glucose, expressed as the area under the curve (AUC). *p<0.01; **p<0.05 vs AL rats (Student's t test).*

ported by analysis of data in Fig. 4, showing a significant decrease of ΔG in $VOSO_4$-treated rats with respect to the age-matched controls. To this regard, it may be noted that because of the tendentiously lower ΔI observed in $VOSO_4$-treated rats, the insulinogenic index ΔI/ΔG was similar in treated and untreated animals (and in both cases significantly lower than that calculated for 16-month-old rats), confirming that the beneficial effect of $VOSO_4$ administration was not related to an insulinotropic effect, but to an improved overall utilization of glucose.

Our results also indicate that dietary restriction was able to significantly reduce glycaemic values with respect to the age-matched AL rats, without any increase in post-loading plasma insulin levels.

Glucose-stimulated insulin output from the isolated perfused pancreas of 18-month-old AL (either untreated or $VOSO_4$-treated) and 40% DR rats is shown in Figure 5. In AL rats, the typical biphasic pattern of insulin release was observed, with a first phase peaking at 3 min after glucose concentration was changed from 2.8 to 16.7 mM. The secretory kinetics was not influenced by the two-month oral $VOSO_4$ administration, whereas the overall insulin output was significantly decreased with respect to the age-matched untreated rats (see insert in Fig. 5). Finally, the perfused pancreas of 18-month-old 40% DR rats, while maintaining the biphasic pattern of insulin release, showed a marked reduction of the overall output with respect to both untreated and $VOSO_4$-treated animals.

DISCUSSION

A reduction in peripheral insulin sensitivity is an early event during the aging process, already leading to glucose intolerance in 6-month-old rats (13). Several studies utilizing the euglycaemic clamp technique indicate that the age-related impairment in glucose disposal may be in large part dependent on the development of peripheral insulin resistance (5, 14, 15). The insulino-mimetic properties of vanadium and its derivatives are well known, including its ability to alleviate some symptoms of experimental diabetes in the rat (16, 17). Although the mechanism of action of vanadium has not been fully clarified, many of its *in vivo* insulin-like effects have been attributed to the ability of restoring peripheral tissue sensitivity to circulating insulin (18). On the other hand, dietary restriction is now largely accepted as an effective tool in delaying the aging process and decreasing the incidence of various tumours and other age-associated diseases (19, 20). In fact, it represents the most effective means of extending lifespan in experimental animals (9) and elicits various biochemical and molecular changes (8).

In view of the considerations above, we explored whether the age-related alterations of glucose homeostasis in rats could be prevented and/or corrected by either a chronic (two months) treatment with oral vanadyl sulfate (0.5 mg/ml in the drinking water) or a long-term dietary restriction. In order to obtain a reliable overall evaluation of the changes in the pancreatic secretory response and the peripheral glucose utilization, intravenous

glucose tolerance tests were carried out longitudinally. As a first observation, our experiments confirm that age-related differences occur in the glycaemic and insulinaemic curves obtained after the glucose load. Indeed, while in young animals a large amount of insulin is secreted in the bloodstream in response to the glucose challenge, so that glycaemia rapidly returns to basal values, in older rats glycaemia remains elevated above basal values for longer times in spite of increased (in 16-month-old rats) or similar (in 18-month-old rats) insulin levels. Thus, both 16- and 18-month-old animals show an altered response to a glucose challenge, which is accentuated in the latter group leading to a significantly decreased glucose tolerance (see Fig. 4), probably as a consequence of the attenuation of the compensatory hyperinsulinaemia observed in the former. The prolonged oral administration of $VOSO_4$ is effective in preventing the deterioration of the glucose tolerance occurring between 16 and 18 months of age. This beneficial effect is obtained in $VOSO_4$-treated animals in the presence of post-loading plasma insulin levels significantly lower than those observed in untreated glucose intolerant rats, in agreement with the above mentioned putative peripheral action of vanadium. Although the exact mechanism of this $VOSO_4$ effect is still unknown, it has been shown that vanadium could block the dephosphorylation of substrates involved in the transduction of the insulin signal, thus prolonging the hormonal action (21, 22).

The chronic administration of compounds containing vanadium has been associated with several negative side effects (23-25). However, the toxicity of vanadium is largely dependent on the animal species, the route of administration and its chemical form (for a recent review see 26). In particular, it has been shown that $VOSO_4$ is remarkably less toxic than other compounds, like e.g. sodium metavanadate or sodium orthovanadate (27). A longitudinal study on the toxicological effects of $VOSO_4$ has been performed on rats treated with different doses (from 0.5 to 1.5 mg/ml) in the drinking water. Some observed negative side effects (weight loss, diarrhoea) were largely prevented by the reduction of the dosage (28). The histological examination of tissues preferentially accumulating vanadium (bone, liver, kidney) showed the absence of significant morphological alterations after 39 days of $VOSO_4$ treatment (23). With regard to the debated anorexic effect of vanadium in relation with its hypoglycaemizing properties in experimental diabetes (29, 30), we would like to underline that no significant changes in food and water intake in $VOSO_4$-treated rats occurred in this study during the whole experimental period (mean individual food intake: controls 30.1±1.96 g/day, $VOSO_4$-treated 26.8±1.41 g/day, NS; mean individual water intake: controls 29.1±2.10 ml/day, $VOSO_4$-treated 26.6±2.74 ml/day, NS).

A major aim of this study was to directly compare the effects of oral $VOSO_4$ administration with those of dietary restriction at a critical age for development of glucose intolerance in Sprague-Dawley rats. Our results show that in 40% DR rats, the impairment in glucose homeostasis observed in AL rats is fully prevented at both 16 and 18 months of age. Similarly to $VOSO_4$ administration, the effect of dietary restriction is not related to an enhanced insulin secretion from the pancreas (post-loading plasma insulin levels being usually lower than in AL rats), but rather to a more effective utilization of glucose by tissues. This is in agreement with previous observations indicating that dietary restriction can increase insulin sensitivity in streptozotocin-diabetic rats (31), enhance insulin-mediated glucose uptake and reduce insulin demand in healthy rats (32, 33).

This interpretation of the effects of both treatments (i.e. $VOSO_4$ and dietary restriction) is further supported by the data obtained *in vitro*. Indeed, in the isolated perfused pancreas of both $VOSO_4$-treated and 40% DR rats, glucose-stimulated insulin output was significantly lower than that of AL controls, in agreement with the results obtained in the perfused pancreas of 10-month-old rats by Cadène et al. (34), who showed that chronic $VOSO_4$ administration was associated with a decreased insulin response to glucose. The low responsiveness to glucose of the perfused pancreas from 40% DR rats should be at least in part related to the overnight fasting period preceding pancreas isolation in these animals, which usually eat all the available food within 6-8 hours.

In conclusion, this work shows that both oral vanadyl sulfate administration and dietary restriction are effective in contrasting the development of glucose intolerance in aging rats, and indicates that in both cases this effect is more related to a sensitization of the insulin action at the level of peripheral tissues than to a direct stimulation of β-cell secretory capabilities.

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