[1] Department of Physiology, School of Dentistry,
University of Zagreb, Gundulićeva 5,
HR-10000 Zagreb, Croatia
[2] Department of Physiology, School of Medicine,
University of Zagreb, Šalata 3, HR-10000 Zagreb, Croatia

Original Scientific Paper
Received: June 4, 2004
Accepted: July 20, 2004

EFFECTS OF VANADATE ON GLUCOSE PRODUCTION IN RAT HEPATOCYTES CULTURED *IN VITRO*

Jagoda Roša[1], Josip Roša[2]

Key words: insulin, glucose production, glycogen, gluconeogenesis, glycogenolysis, vanadate

SUMMARY

The aim of the study was to determine the effect of vanadate on glucose production in hepatocytes cultured in vitro*. Hepatocytes were isolated by the collagenase perfusion technique and cultured for 24 h in M 199 serum-free medium. The glucose production was measured by incubating the cultures in glucose-free Hanks-Hepes medium with the addition of 10 mmol/l pyruvate. The glucose released into the medium was determined enzymatically with glucose oxidase. Glycogenolysis was determined as net decrease in glycogen content during the incubation period. Vanadate alone in a concentration of 1 mmol/l caused a significant (p<0.01) 57% increase of glucose production during the first hour, whereafter complete cessation of glucose production occurred and hepatocytes started to consume glucose, shown as a steady and slow glucose decrease in the medium. Although insulin alone did not change glucose production, vanadate in the presence of insulin induced an even stronger (80%) increase in glucose production during the first hour, followed by a very strong (60%) glucose decrease in the medium during the second hour. The results obtained indicated that vanadate decreased glucose production by inhibiting gluconeogenesis.*

INTRODUCTION

The trace element vanadium, widely distributed in nature, has been shown to exert both insulin-like and non-insulin-like action (1). Vanadyl treatment improves glucose homeostasis *in vivo* in several models of diabetes, normalizes blood glucose levels in streptozotocin diabetic rats, and also restores expression of the insulin responsive glucose transport in skeletal muscle (2,3). Vanadate caused longterm and marked improvement of glucose homeostasis in obese fa/fa rats and in diabetic ob/ob mice, and also improved hepatic and peripheral insulin sensitivity in patients with non-insulin dependent diabetes mellitus (4-6). These apparently beneficial effects on peripheral glucose utilization are in contrast with vanadate effect on hepatic carbohydrate metabolism in normal rat, causing increase of hepatic glucose production as well as stimulation of glycogen phosphorylase activity (7,8). Similar activation of glycogen phosphorylase by vanadate was observed in hepatocytes isolated from diabetic rat (9). The prolonged and uncontrolled diabetes is presumably predominated by an increase in gluconeogenesis and overproduction of glucose from the liver, therefore it would be of interest to find out the effect of vanadate on this process (10). In this study, direct effects of vanadate on glycogenolysis and gluconeogenesis were investigated in hepatocytes cultured *in vitro*.

MATERIAL AND METHODS

Animals

Adult male Wistar rats weighing 250-325 g were used in the experiments. Rats were housed individually in wire cages in a temperature-controlled room (21±1 °C), on 12 h light-dark cycle, with free access to food and water. The principles of animal care (NIH publication No. 85-23, revised 1985) were followed.

Hepatocyte culture preparation

Hepatocytes were isolated by a modified collagenase-perfusion technique (11). The rats were anesthetized with phenobarbital (10 mg/100 g body weight) and calcium-free Swim's S-77 medium containing collagenase (0.5 g/l were used for liver perfusion through a portal cannula). Usually more than 90% of cells excluded trypan blue as the measure of viability. After washing twice with the same collagenase-free medium, the cells were suspended to a final concentration of one million cells *per* ml M199 serum-free medium. Three ml of cell suspension were placed in 60-mm Petri dishes previously coated with collagen. Culture dishes were kept at 37 °C in an atmosphere of 5% CO_2 and 95% air (Heraeus CO_2 incubator, Hanau, Germany). The culture medium was replaced with fresh medium 4 hours later to remove unattached cells and hepatocytes were incubated for the next 24 h in the M199 serum-free medium.

Glucose production

After having been left for 24 h in culture, the medium was removed and cells were incubated in glucose-free Hanks-Hepes medium containing 10 mmol/l pyruvate, without hormones (control) or insulin (80 nmol/l) or vanadate (1 mmol/l). The glucose released into the medium was determined enzymatically with glucose oxidase. The glucose production was measured by incubating the cultures in glucose-free Hanks-Hepes medium with the addition of 10 mmol/l pyruvate. The incubation medium was removed and hepatocytes were washed three times with cold saline and frozen immediately in liquid nitrogen. The cells were digested in 0.2 N NaOH and an aliquot was taken for glycogen and protein determination. The amount of glycogen was determined by the method of Roehring and Allred (12), and protein by the method of Lowry *et al*. (13).

Chemicals

Bovine albumin, glutamine, HEPES, M199 medium, Swim's S-77 medium, insulin and vanadate were obtained from Sigma; Collagenase CLS II (131U/ mg) was purchased from Worthington; Collagen R was purchased from Serva.

Perfusion medium is a Swim's S-77 medium containing 2.2 g $NaHCO_3$ and 585 mg glutamine *per* liter.

Incubation medium is a M199 medium containing the following additions *per* liter: 2 g albumin, 900 mg L-glutamine and 2.2 g $NaHCO_3$.

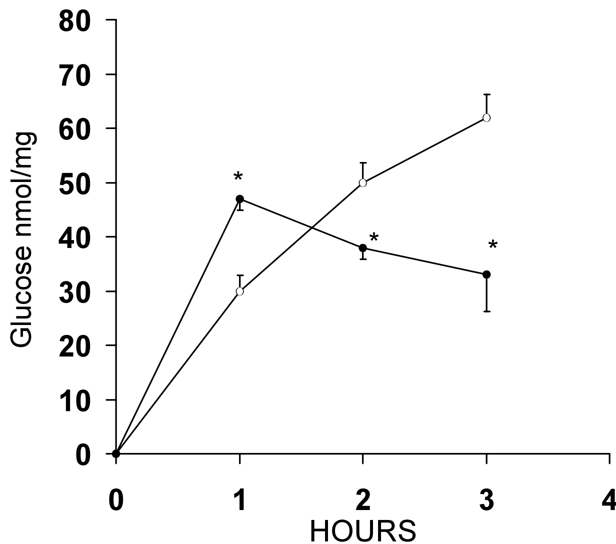
Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was evaluated by Student's t-test; $p<0.01$ was considered statistically significant.

RESULTS

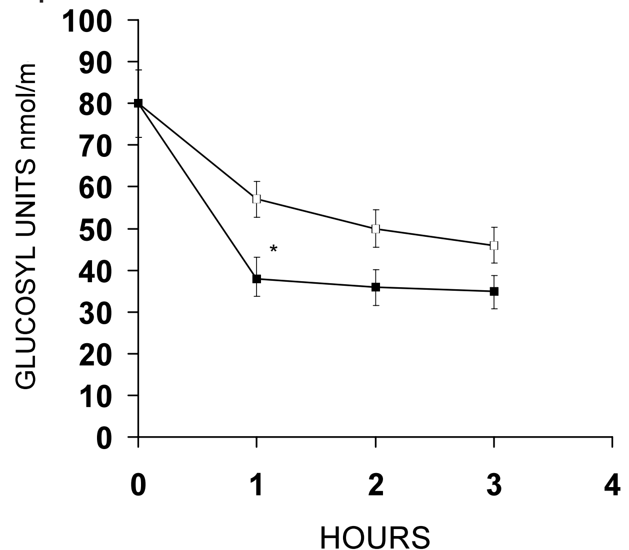
The rate of glucose production in glucose-free Hanks Hepes medium in the presence of pyruvate showed two components. The highest glucose production was recorded during the first hour, followed by a decrease in the second and third hour (Fig. 1). The major part of glucose produced during the first hour of incubation was delivered by quick activation of glycogenolysis. After the cell incubation in glucose-free Hanks-Hepes medium, there was a rapid and very great decrease in glycogen concentration. The highest rate of glycogenolysis in control cultures was in the first hour, whereas a considerably lower rate was recorded during the second hour and complete cessation in the third hour (Fig. 2). Vanadate alone in a concentration of 1 mmol/l caused a significant (57%) increase in glucose production during the first hour, whereafter complete cessation of glucose production occurred and hepatocytes started to consume glucose, shown as a steady and slow decrease of glucose in the medium (Fig. 1). In cultures treated with vanadate and insulin

Figure 1. **Glucose production (nmol/mg protein) in cultured hepatocytes incubated in glucose-free Hanks-Hepes medium in the presence of 10 mmol/l pyruvate and treated with 1 mmol/l vanadate (-●-) and untreated controls (-❍-). Each point is mean ± SEM for five plates in two separates experiments.**



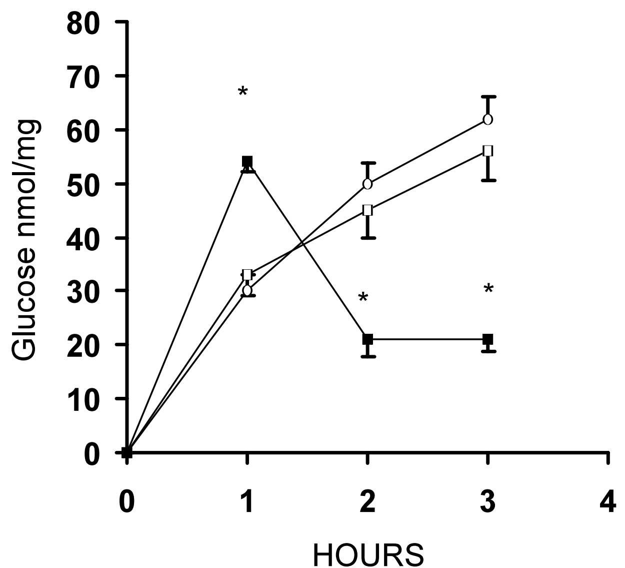
* p<0.01 compared with untreated controls.

Figure 2. **Glycogen concentration (glycosyl units nmol/mg protein) in cultured hepatocytes incubated in glucose-free Hanks-Hepes medium in the presence of 10 mmol/l pyruvate and treated with 1 mmol/l vanadate (-■-) and untreated controls (-❑-). Each point is mean ± SEM for five plates in two separates experiments.**



* p<0.01 compared with untreated controls.

Figure 3. **Glucose production (nmol/mg protein) in cultured hepatocytes incubated in glucose-free Hanks-Hepes medium in the presence of 10 mmol/l pyruvate and treated with 80 nmol/l insulin (-❑-), insulin (80 nmol/l) and vanadate (1 mmol/l) (-■-) and untreated controls (-❍-). Each point is mean ± SEM for five plates in two separates experiments.**



* p<0.01 compared with untreated controls.

together there was a significantly higher increase of glucose production during the first hour, followed by very strong glucose reduction in the medium during the second hour, although insulin alone caused no significant changes in glucose production throughout the three-hour period (Fig. 3).

DISCUSSION

The present study demonstrated that vanadate increased glucose production only during the first hour, when glycogenolysis was activated in the glucose-free medium. Rapid release of glucose into the medium corresponded with the decrease in cellular glycogen. These results are in agreement with the report of Bosh *et al*. (8), who demonstrated glycogenolytic effect of vanadate in freshly isolated hepatocytes. The activation of glycogen phosphorylase was also detected in isolated hepatocytes of streptozotocin-diabetic rat (9). Interestingly, we demonstrated a similar glycogenolytic effect in animals after insulin injection. Insulin injection causes deep hypoglycemia, which in turn induces intense glycogenolysis in the animal liver

(14). In our experiments, intense glycogenolysis was activated in glucose-free medium, however, insulin did not cause any significant increase in glucose production during the first hour in glucose-free medium. On the other hand, vanadate in the presence of insulin caused an even stronger increase in glycogenolysis during the first hour (Fig. 3). In contrast to our results, Roden *et al.* found that insulin completely blocked glycogenolytic effect of vanadate in isolated livers of non-fasting rats (7). They perfused livers with a medium containing 5 mmol/l glucose, whereas we used glucose-free medium, which might possibly explain the differences.

When blood glucose concentration is low *in vivo*, or glucose-free medium *in vitro*, hepatocytes produce glucose mainly from glycogen, which we also demonstrated during the first hour of incubation. Glycogen stores are limited and exhausted after one hour (Fig 2). Then glucose supply relies on its synthesis from non-glucidic precursors such as lactate or pyruvate (15). In our study, the process of gluconeogenesis was activated during the first hour of incubation, to become the main process for glucose production during the second and third hour. Vanadate alone as well as in the presence of insulin completely blocked this process. Hepatocytes started consuming glucose rather than producing it (Figs. 1 and 3). This process was especially intensive during the second hour in cultures treated with vanadate and insulin together (Fig. 3).

We have already shown that the glycogen content in hepatocytes of acute alloxan-diabetic rats is rather low, and in chronically alloxan-diabetic animals very low (16). Similar glycogen depletion in the myocardium, muscle and liver of diabetic rats has been described by others (17,18). It is not clear whether the decreased amount of glycogen was the result of a defect in the hepatic storage capacity or of impaired synthetic processes. In the states in which glycogen is low, such as diabetes, the glycogenolytic effect of vanadate contributes very little to diabetic hyperglycemia. On the other hand, the finding that vanadate inhibited gluconeogenesis in the liver, a process that predominates in prolonged and uncontrolled diabetes, clearly demonstrated its possible beneficial effect in therapy of diabetes.

Acknowledgments. We thank Mr. Boris Tomašević for his expert technical assistance. This study was supported by the grant No. 0065002 from the Croatian Ministry of Science and Technology.

REFERENCES

1. Roša J, Roša J. Insulin-like and non-insulin-like action of vanadate in hepatocytes cultured *in vitro*. Coll Antropol 2004;28:309-315.

2. Meyerovitch J, Farfel Z, Sack J, Shechter Y. Oral administration of vanadate normalises blood glucose levels in streptozotocin-treated rats, characterisation and mode of action. J Biol Chem 1987;262:6658-6662.

3. Strout HV, Vicario PP, Biswas C, Saperstein R, Brady EJ, Pilch PF, Berger J. Vanadate treatment of streptozotocin diabetic rats restores expression of the insulin-responsive glucose transporter in skeletal muscle. Endocrinology 1990;126:2728-2732.

4. Brichard SM, Potter AM, Henquin JC. Long term improvement of glucose homeostasis by vanadate in obese hyperinsulinemic fa/fa rats. Endocrinology 1989;125:2510-2516.

5. Brichard SM, Bailey CJ, Henquin JC. Marked improvement of glucose homeostasis in diabetic ob/ob mice given oral vanadate. Diabetes 1990;39:1326-1332.

6. Cohen N, Halberstam M, Shlimovich P, Chang CJ, Shamoon H, Rossetti L. Oral vanadyl sulphate improves hepatic and peripheral insulin sensitivity in patients with non-insulin-dependent diabetes mellitus. J Clin Invest 1995;95:2501-2509.

7. Roden M, Liener K, Fürnsinn C, Prskavec M, Nowotny P, Steffan I, Vierhapper H, Waldhäusl W. Non-insulin-like action of sodium orthovanadate in the isolated perfused liver of fed, non-diabetic rats. Diabetologia 1993;36:602-607.
8. Bosch F, Arino J, Gomez-Foix AM, Guinovart JJ. Glycogenolytic, noninsulin-like effects of vanadate on rat hepatocyte glycogen synthase phosphorylase. J Biol Chem 1987;262:218-222.
9. Rodriquez-Gil JE, Gomez-Foix AM, Arino J, Gunovart JJ, Bosch F. Control of glycogen synthase and phosphorylase in hepatocytes from diabetic rats. Diabetes 1989;38:793-798.
10. Unger RH. Role of glucagon in the pathogenesis of diabetes: the status of the controversy. Metabolism 1978;27:1691-1709.
11. Rosa J, Rubin E. Effects of ethanol on amino acid uptake by rat liver cells. Lab Invest 1980;43:366-372.
12. Roehring KL, Allred JB. Direct enzymatic procedure for determination of glycogen. Anal Biochem 1974;58:414-421.
13. Lowry OH, Rosenbrough NJ, Farr AL, Randel RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265-271.
14. Hers HG. The control of glycogen metabolism in the liver. Ann Rev Biochem 1976;45:167-189.
15. Hers HG, Hue L. Gluconeogenesis and related aspects of glycolysis. Ann Rev Biochem 1983;52:617-653.
16. Roša J, Soljačić M, Slijepčević M, Roša J. Insulin sensitivity in hepatocytes cultured from normal and alloxan-diabetic rats. Diabetol Croat 1994;23:139-146.
17. Shaffer SW, Seyed-Mozaffari M, Cutcliff CR, Wilson GL. Post-receptor myocardial metabolic defect in a rat model of non-insulin-dependent diabetes mellitus. Diabetes 1986;35:593-597.
18. Kruszynska YT, Home PD. Liver and muscle insulin sensitivity, glycogen concentration and glycogen synthase activity in a rat model of non-insulin-dependent diabetes. Diabetologia 1988;31:304-309.