

VANADATE MODULATES HORMONAL REGULATION OF GLUCOSE PRODUCTION IN CULTURED HEPATOCYTES ISOLATED FROM RATS ON HIGH UNSATURATED FAT DIET

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Vanadate showed an insulinomimetic effect acting directly on basal as well as on glucagon-stimulated glucose production in hepatocytes, probably inhibiting gluconeogenesis.

SUMMARY

Insulin resistance is a common phenomenon in obesity and type 2 diabetes. Increased dietary fat will lead to impairment of insulin action. Vanadate treatment improves glucose homeostasis in vivo. The aim of this study was to show changes in hepatic glucose output in dependence of fat diet and the possible direct action of vanadate on cultured hepatocytes. Hepatocytes were isolated by the collagenase perfusion technique and cultured for 24 h in M 199 serum-free medium. 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. Glucose production in hepatocytes isolated from rats on high fat diet was significantly ($p < 0.01$), i.e. by 79% higher as compared with controls on standard diet. The addition of vanadate (1 mmol/l) completely normalized glucose production. Glucagon (0.2 μ mol/l) significantly ($p < 0.01$), i.e. by 150% increased glucose production in hepatocytes from rats on standard diet and by 226% in hepatocytes from rats on high fat diet. Vanadate (1 mmol/l) significantly ($p < 0.01$), i.e. by 50% decreased glucagon-stimulated glucose production and presence of insulin influenced neither the glucagon nor the vanadate effect.

INTRODUCTION

A high level of dietary fat intake in the Western diet is believed to be a major factor in the development of obesity and insulin resistance (1,2). Increased fat intake also appears to lead to insulin resistance independent of adiposity (3). Rats on high fat diet had a greater hepatic glucose production compared with rats on standard diet (4). Increased hepatic glucose production correlates well with fasting glucose levels and is the main cause of fasting hyperglycemia in type 2 diabetes (3). Besides this, it is characterized by hormonal imbalance, the result of prevailing hyperglucagonemia accompanied by insulin resistance. Prolonged and uncontrolled diabetes is presumably predominated by an increase in gluconeogenesis and overproduction of glucose from the liver (5). The trace element vanadium is widely distributed in nature and has been shown to have an insulin-like action (6). Vanadate caused longterm and marked improvement of glucose homeostasis in diabetic ob/ob mice and in obese fa/fa rats (7,8). Vanadate initially increased and later decreased glucose production probably by inhibiting gluconeogenesis in cultured hepatocytes isolated from rats on standard food (4). In this study we

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tried to show changes in hepatic glucose output in dependence of fat diet and the possible direct action of vanadate on cultured hepatocytes.

MATERIAL AND METHODS

Animals

In all experiments, male adult Wistar rats weighing 250-325 g were used. Rats were housed individually in wire cages in a temperature-controlled room ($21 \pm 1^\circ\text{C}$), on a 12-h light-dark cycle, with free access to food and water for three weeks. High fat diet was prepared by admixing 30% of sunflower oil to standard food. High fat diet contained 30% carbohydrates, 16% protein and 54% fat, as previously reported (4).

The principles of animal care (NIH publication No. 85-23, revised 1985) were followed during the experiments.

Preparation of hepatocyte culture

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 hours in the M199 serum-free medium.

Glucose production

After having been left in culture for 24 h, the medium was removed and cells were incubated in glucose-free Hanks-Hepes medium containing 10 mmol/l pyruvate, without hormones (control), or with insulin (80 nmol/l), glucagon (0.2 $\mu\text{mol/L}$) or vanadate (1 mmol/l).

The glucose released into the medium was determined enzymatically with glucose oxidase. 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 the determination of protein.

Chemicals

Albumin bovine, 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 was a Swim's S-77 medium containing 2.2 g NaHCO_3 and 585 mg glutamine *per* liter.

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

Statistical analysis

Data are expressed as means \pm SEM. Statistical significance was evaluated by Student's t-test. Statistical significance was set at $p < 0.01$.

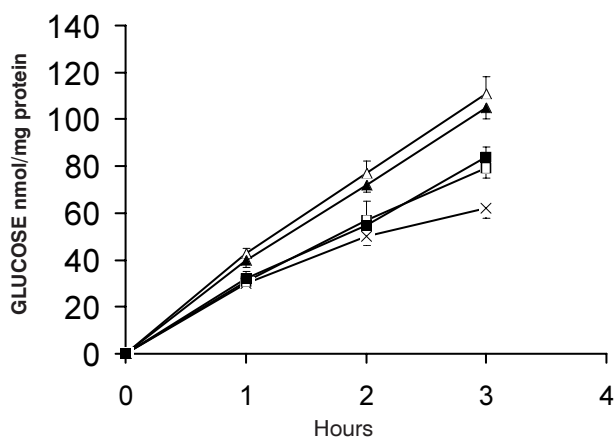
RESULTS

The highest rate of glucose production in control cultures was recorded in the first hour, whereas a considerably lower rate was found during the second and third hour.

In cultures isolated from rats on high fat diet there was an almost linear increase in glucose production throughout the 3-hour period, exceeding that in control cultures by 25% in the first hour, by 54% in the second hour, and by 79% in the third hour. Insulin alone, in a concentration of 80 nmol/l, did not change glucose production in hepatocytes isolated from rats on high fat diet, which remained higher by 69% compared with standard cultures after 3-hour period (Fig. 1). The addition of vanadate (1 mmol/l) completely normalized glucose production in the first and second hour,

however, after third hour it still minimally (27%) exceeded that in standard cultures. The effect of vanadate remained unchanged in the presence of insulin (Fig. 1).

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, isolated from rats on sunflower oil diet without (-△-) or treated with insulin (80 nmol/l) (-▲-), or vanadate (1 mmol/l) (-□-), or insulin and vanadate (-■-), and standard control (-x-). Each point is the mean ± SEM of five plates in two separate experiments.



Glucagon increased glucose production during the first and second hour by 100%, the increase reaching as high as 150% in the last hour in control group. In cultures isolated from rats on high fat diet the glucagon induced increase was much higher, i.e. 140% after the first hour, 180% after the second hour, and even 226% after the third hour. As the result of such a great increase in glucose production, the glucose released in the medium at the end of the 3-hour period was by 137% higher in cultures isolated from rats on high fat diet compared with standard control (Fig. 2). Vanadate treatment could not normalize this tremendous increase in glucose production induced by glucagon. In the presence of vanadate the glucagon induced increase was by 50% lower, yet the glucose production exceeded that in standard control by 100% (Fig. 3). The presence of insulin influenced neither the glucagon nor the vanadate effect (Fig. 3).

Figure 2. Glucose production (nmol/mg protein) in cultured hepatocytes incubated in glucose-free Hanks-Hepes medium in the presence of 10 mmol/l pyruvate, isolated from rats on sunflower oil diet without (-△-) or treated with glucagon (0.2 μmol/l) (-▲-) compared with production from rats on standard diet without (-□-) or treated with glucagon (0.2 μmol/l) (-■-). Each point is the mean ± SEM of five plates in two separate experiments.

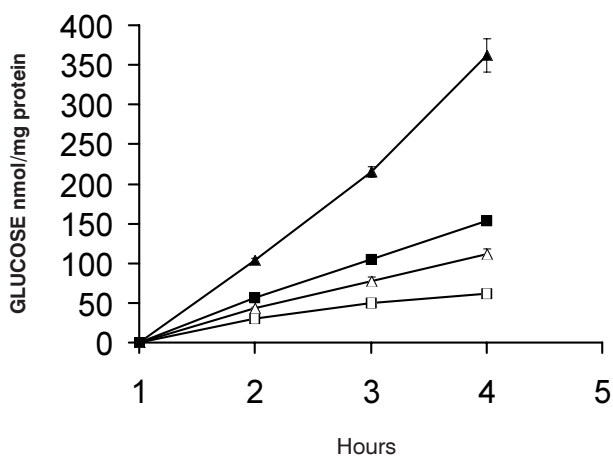
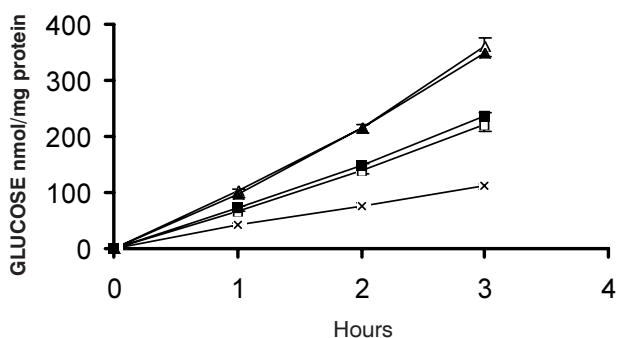


Figure 3. Glucagon stimulated glucose production (nmol/mg protein) isolated from rats on sunflower oil diet without (-△-) or treated with insulin (80 nmol/l) (-▲-) or vanadate (1 mmol/l) (-□-) or insulin and vanadate (-■-), and standard control (-x-). Each point is the mean ± SEM of five plates in two separate experiments.



DISCUSSION

The present study demonstrated the basic glucose production and even more the glucagon stimulated glucose production in hepatocytes cultured *in vitro* to be highly dependent on the percentage of fat consumed during a three-week period in rats. We have previously shown and now confirmed the basic glucose production in hepatocytes isolated from rats on high fat diet to be significantly ($p < 0.01$) higher compared with standard control (4). What is more important, glucagon induced a 226% increase of glucose production after a 3-hour period (Fig. 2). Such a tremendous increase in hepatic glucose production could be a major cause of fasting hyperglycemia in prediabetic state as well as in type 2 diabetes (3). Hepatic glucose production is regulated by a number of factors, including hormones, substrates and neural stimulation (10). Hepatic glucose production represents a sum of the glucose derived from glycogen stores and that originating from gluconeogenesis. When blood glucose concentration is low *in vivo*, or in glucose-free medium *in vitro*, hepatocytes produce glucose mainly from glycogen. The glycogen stores are limited and exhausted after one hour in hepatocytes *in vitro* (11). Then glucose supply relies on its synthesis from non-glucidic precursors such as lactate or pyruvate (12). The process of gluconeogenesis was activated during the first hour of incubation in hepatocytes, and it became the main process for glucose production during the second and third hour (11). High hepatic free fatty acid oxidation promotes gluconeogenesis *via* production of ATP, NADH, and acetyl-CoA (13). All these factors together with higher sensitivity to glucagon or permissive action

of some other factors like glucocorticoids could explain such a tremendous increase of glucose production in our experiments.

Another very important finding was the failure of insulin to inhibit glucose production (Figs. 1 and 2). This could be the result of insulin resistance developing after three-week high fat feeding. Some *in vivo* as well as *in vitro* studies have suggested that much of insulin action on the liver may be indirect and related to systemic rather than hepatic effects (14,15). Direct insulin effects on glucose production in the liver, especially on gluconeogenesis, are highly dependent on the level of glucose in the medium. In all our experiments glucose production was measured in a glucose free medium and this could explain our finding that insulin did not affect glucose production, although insulin resistance could also be a contributing factor.

Vanadate alone as well as in the presence of insulin completely normalized basic glucose production (Fig. 1). We have already shown that vanadate inhibited gluconeogenesis in the liver (11). This is especially important in conditions where the process of gluconeogenesis is very active, like in animals held on high fat diet. The finding that vanadate also partially prevented glucagon stimulated glucose production clearly demonstrated its possible beneficial effect in the prevention and therapy of diabetes.

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