SUMMARY

The aim of the study was to elucidate the mechanism of oleic acid induced insulin resistance and vanadate action on amino acid transport in cultured rat hepatocytes. Hepatocytes were isolated by a modified collagenase perfusion technique and cultured for 24 h in M199 serum-free medium. To assess amino acid transport, hepatocytes were incubated in Hanks-Hepes medium containing α-amino14C-isobutyric acid (AIB) to the final concentration of 1 mmol/L. In hepatocytes obtained from rats on standard diet, insulin almost two fold increased AIB transport (5.57±0.31 vs. 10.15±0.21; p<0.01). Oleic acid did not change basal AIB transport but reduced insulin effect to less than a half value (5.36±0.27 vs. 6.25±0.19; p<0.01). Vanadate increased basal (5.57±0.3 vs. 10.97±0.35; p<0.01) and did not change insulin stimulated (10.15±0.21 vs. 11.23±0.45; N.S.) AIB transport in normal hepatocytes. It normalized insulin stimulated transport in oleic acid induced resistant hepatocytes (5.36±0.27 vs. 11.25±0.19; p<0.01) but not in TPA treated (6.82±0.2 vs. 6.7±0.25; N.S.) resistant cells. Vanadate was able to prevent and reverse insulin resistance induced by oleic acid not involving protein kinase C.

INTRODUCTION

Insulin resistance is a common phenomenon in obesity and type 2 diabetes (1). It has been demonstrated that, among other factors, acutely elevated free fatty acids can induce insulin resistance in both peripheral tissues and liver (2). Free fatty acids also reduce insulin binding and degradation, and exert an important modulating effect on insulin action in isolated rat hepatocytes (3). They can also increase hepatic gluconeogenesis (4). Vanadate caused longterm and marked improvement of glucose homeostasis in obese fa/fa rats and diabetic ob/ob mice (5,6). Vanadate also improved hepatic and peripheral insulin sensitivity in patients with non-insulin dependent diabetes mellitus (7). However, these results are in contrast to the finding that acute infusion of vanadate stimulates glucose production in perfused livers from fed rats (8). Besides these metabolic effects, vanadate-dependent inhibition of phosphatase, increased insulin binding to target cells and enhanced tyrosine phosphorylation of insulin receptor have also been reported, all suggesting the possible modulatory action of vanadate on insulin signaling (9,10). This could be the main way of vanadate action on insulin resistance and glucose homeostasis. The aim of the study was to investigate this hypothesis by studying the effects of vanadate on amino acid transport in cultured rat hepatocytes, which became insulin-resistant after acute oleic acid treatment.
MATERIAL AND METHODS

Materials

Alpha-amino$^{14}$C-isobutyric acid (AIB) (1.85-2.29 GBq/mmol) was purchased from The Radiochemical Center, Amersham; bovine albumin, glutamine, HEPES, M199 medium, Swim’s S-77 medium, insulin, sodium orthovanadate (vanadate), phorbol 12-myristate 13-acetate and polymyxin B 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$_3$.

Animals

Adult male Wistar rats (bred at our facility) were fed standard (4% fat) diet. Rats were housed individually in wire cages in a temperature-controlled room (21°C), at 12-h light-dark cycle, with free access to food and water. The principles of animal care (NIH publication No. 85-23, 1985 revision) were followed.

Hepatocyte culture

Hepatocytes were isolated by a modified collagenase-perfusion technique (11). The rats were anesthetized with phenobarbital and calcium-free Swim’s S-77 medium containing collagenase (0.5 g/L) was used for liver perfusion through a portal cannula. Usually more than 90% of cells excluded trypan blue as a 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 (CO$_2$ incubator Heraeus, 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.

Amino acid transport

Hepatocytes were preincubated for 4 h in the absence and presence of 80 nmol/L insulin, or 0.4 mmol/L oleic acid, or 1 mmol/L vanadate, or 20 mmol/L polymyxin B, or 0.1 mmol/L phorbol 12-myristate 13-acetate (TPA). After the preincubation period, the medium was removed and hepatocytes were incubated in Hanks-Hepes medium containing 2 g/L albumin and α-amino$^{14}$C-isobutyric acid (AIB) to the final concentration of 1 mmol/L. One hour later, the incubation was terminated by removing the medium and hepatocytes were quickly frozen in liquid nitrogen after three washes with cold saline. The cells were digested in 0.2 mol/L NaOH and aliquots were taken for determination of protein (Spectrophotometer Novaspec II, Amersham Pharmacia Biotech, Cambridge, UK) and radioactivity in a liquid scintillation counter (Beckman LS-250, Fullerton, USA).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was evaluated by Student’s t-test. Statistical significance was set at p<0.05.

RESULTS

The induction of AIB transport by insulin in hepatocytes is shown in Figure 1. The 4-hour pretreatment with insulin led to a significant (p<0.01), almost twofold increase in AIB transport. The level of insulin used was 80 nmol/L, at which maximal transport stimulation was observed. In the presence of 0.4 mmol/L oleic acid the basal AIB transport did not change significantly. Vanadate, when added alone over a 4-hour pretreatment period, stimulated AIB transport even slightly stronger than insulin (p<0.01). The concentration of vanadate used was 1 mmol/L, which has already been found optimal in cultured hepatocytes. The addition of insulin together with vanadate over the 4-hour pretreatment period did not produce any additional effect but increased AIB transport like in the cultures pretreated with insulin or vanadate alone (p<0.01) (Fig. 1). However, in the presence of oleic acid the maximal insulin effect was significantly reduced to less than a half. On the other hand, the effect of vanadate remained unchanged in
cultures pretreated with vanadate alone or in those pretreated with vanadate and insulin together (Fig. 1). The biologic effects of insulin are mediated through activation of the insulin receptor tyrosine kinase. A number of different factors can modulate the kinase activity, and protein kinase C (PKC) has been shown to play a pivotal role. So, we treated cells with TPA and polymyxin B, a stimulator and an inhibitor of PKC activity. TPA significantly and slightly stimulated basal and insulin stimulated transport, respectively, and significantly reduced the effect of vanadate (Fig. 2). On the other hand, polymyxin B partially antagonized the oleic acid induced insulin resistance, whereas the effect of vanadate remained unchanged (Fig. 3).

DISCUSSION

In the present study, we evaluated the impact of vanadate on insulin resistance induced in vitro by oleic acid during a 4-hour pretreatment period. The oleic acid induced insulin resistance is an acute and reversible process with possible changes in the insulin receptor kinase activity involving PKC (12). When
added alone over a 4-hour pretreatment period, vanadate stimulated AIB transport like insulin, and the addition of insulin along with vanadate did not produce any extra effect. This insulin-like effect of vanadate could be the result of enhanced tyrosine phosphorylation of the insulin receptor, or inhibition of phosphatase (9,10). There is also another possibility that the insulin-like effect of vanadate is not facilitated through the insulin receptor tyrosine kinase, but by some other cytosolic or membranous nonreceptor protein tyrosine kinase, as demonstrated in rat adipocytes (13). This second possibility could explain how vanadate overcame insulin resistance induced by oleic acid in our experiments, as it utilizes alternative (insulin-independent) mechanisms to manifest its insulin-like effect. This effect of vanadate on the basal AIB transport partially disappeared in hepatocytes pretreated with TPA. PKC stimulating phorbol esters are able to induce in vitro insulin resistance of isolated adipocytes, hepatocytes and Fao-hepatoma cells (14-16). It has been reported that phorbol esters stimulation leads to PKC activation and serine phosphorylation of the insulin receptor β subunit (16). The activation of PKC could also affect alternative insulin-independent mechanisms, or some other, unknown mechanism was involved.

Insulin resistance is a primary feature of type 2 diabetes, and vanadate treatment improves peripheral and hepatic insulin sensitivity in vivo. An alternative insulin-independent mechanism, which overcomes insulin resistance, might be the route of vanadate action.

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

REFERENCES


