Effects of Tumor Necrosis Factor-α on Insulin Stimulated Amino Acid Transport in Cultured Rat Hepatocytes

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ABSTRACT

It has been suggested that tumor necrosis factor alpha (TNF-α) plays a pivotal role in the pathogenesis of insulin resistance. It could act directly or indirectly in liver. The aim of this study was to determine direct short time (4 h) and long time (24 h) action of TNF-α on amino acid transport in cultured rat hepatocytes and possible role of protein kinase C (PKC) in insulin signal pathway and insulin resistance. Hepatocytes were isolated by a modified collagenase perfusion technique and cultured for 24 h in M 199 medium. In the presence of insulin basal alpha-amino isobutyric acid (AIB) uptake was increased 55%. TNF-α in short time action did not change basal AIB transport, but significantly (25%) increased insulin stimulated uptake. Short time action of TNF-α was ameliorated by phorbol ester treatment. These results indicated that PKC activation is important in insulin signaling and TNF-α action. TNF-α acting directly did not cause insulin resistance in cultured hepatocytes.

Key words: amino acid transport, insulin, insulin resistance, protein kinase C, TNF-α

Introduction

Insulin resistance is a component of the metabolic syndrome associated with obesity. A combination of insulin resistance and pancreatic beta-cell dysfunction underlies most cases of type 2 diabetes. Insulin resistance is also associated with a variety of pathological conditions, including trauma, infection and cancer. Among a number of substances, which might play a role in the development insulin resistance in so various pathological conditions, the most interesting is TNF-α. Recent data have revealed that inflammation is a link between insulin resistance, obesity and diabetes. These conditions are associated with a state of abnormal inflammatory response at metabolically relevant sites such as liver, muscle and adipose tissues, which can alleviate insulin resistance. Obese animals with insulin resistance and type 2 diabetes express elevated levels of TNF-α. In vivo neutralization of TNF-α dramatically improves the sensitivity of these obese-diabetic animals, indicating that TNF-α may be a key mediator of insulin resistance in type 2 diabetes. The mechanism by which TNF-α induces insulin resistance in whole animals is not clear. Generally speaking, it can act directly or indirectly through another factor that is released in circulation and inhibit insulin action. The purpose of the present study was to investigate the possible direct effects of TNF-α in hepatocytes cultured in vitro. Transport across the plasma membrane is the first step in amino acid metabolism. The so called «A» system or alanine-preferring system is regulated by hormones and is effectively studied using AIB, which is a non-metabolizing analog of alanine. Insulin increased both the initial rate of AIB uptake and a total amount taken up at later time intervals and we showed that free fatty acids produce post binding perturbations finally leading to reduction of insulin effect. The aim of this study was to determine direct short time and long time action of TNF-α on amino acid transport in cultured rat hepatocytes and possible role of PKC in insulin signal pathway and insulin resistance.
Materials and Methods

In all experiments, male adult Wistar rats, each weighting 187–240 g, were used. 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.

Hepatocytes were isolated by a modified collagenase-perfusion technique. The rats were anaesthetized with Phenobarbital (10 mg/100 g body weight) 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 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 was placed in 60-mm Petri dishes previously coated with collagen. Culture dishes were kept at 37 °C in an atmosphere of 5% CO2 and 95% air (CO2 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.

For the measurement of alfa-amino isobutyric acid (AIB) transport, the medium was removed and hepatocytes were incubated in Hanks-Hepes medium containing 1 mmol/l 14C-AIB, without hormones (control) or in presence of TPA (0.1 mmol/l). One hour later, the incubation medium was removed and after three washes with cold saline, hepatocytes were frozen immediately in liquid nitrogen. The cells were digested in 0.2 N NaOH and an aliquot was taken for the determination of protein as well as determination of radioactivity in a liquid scintillation counter.

Albumin bovine, glutamine, TNF-α, HEPES, M199 medium, Swim's S-77 medium, insulin, 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 NaHCO3 and 585 mg glutamine per litter. The data are expressed as means ± SEM. Statistical significance was evaluated by Student’s t-test. p<0.05 was considered statistically significant.

Results

The induction of AIB transport by insulin in hepatocytes short time pre-treated with hormone is shown on Figure 1. After short time (4 hours) of pre-incubation with insulin there was significant (p<0.01), 55% increase in AIB transport. TNF-α when added alone during short time pre-incubation period had no effect on AIB transport. However short time pre-treatment with TNF-α significantly increased insulin stimulated transport for 25%. Pre-treatment with TPA significantly decreased insulin stimulated transport and totally abolished TNF-α effect (Figure 1).

After long time pre-treatment (24 hours) with insulin there was significant (p<0.01), 64% increase in AIB transport. TNF-α when added alone or together with insulin during long time pre-incubation had no effect on AIB transport (Figure 2).

![Fig. 1. Short time effect of TNF-α, TPA and insulin on amino acid transport. Hepatocytes were isolated from rats on standard diet and cultured 24 hours in M199 medium. Hepatocytes were pre-incubated 4 hours without or with TNF-α (17 μg/l) or with insulin (80 nmol/l) or both and than incubated as described in the method section. Results are expressed as nmol AIB transported in 1 hour per mg of protein. Each value is the mean ± SEM of 9 determination from 3 separate experiments.](image1)

![Fig. 2. Long time effect of TNF-α and insulin on amino acid transport. Hepatocytes were isolated from rats on standard diet and cultured 24 hours in M199 medium. Hepatocytes were pre-incubated 24 hours without or with TNF-α (17 μg/l) or with insulin (80 nmol/l) or both and than incubated as described in the method section. Results are expressed as nmol AIB transported in 1 hour per mg of protein. Each value is the mean ± SEM of 9 determination from 3 separate experiments.](image2)
Discussion

The present study demonstrates that TNF-α, when added alone did not affect basic AIB transport, but act permissively and significantly increased insulin stimulated transport. This effect was manifested only during short time pre-treatment period. Direct action of TNF-α in hepatocytes did not suggest that it play directly role in the pathogenesis of insulin resistance in liver. Some in vivo as well as in vitro studies showed that at least 3 days of TNF-α treatment are needed to observe alteration in insulin action10,11. Previous studies on cultured adipocytes have suggested that the down regulation of the insulin-regulated glucose transporter (Glut 4) mRNA might be a mechanism of TNF-α mediated insulin resistance and it could be result of »dedifferentiation« of adipocytes11. Some other studies showed that TNF-α act more specifically interfering with insulin signaling in various cell lines or primary skeletal muscle cultures10,12. TNF-α caused tyrosine phosphorylation and activation PKCα and δ in the primary cultures of mouse skeletal muscle12. We pre-treated cells with TPA which stimulate PKC activity of certain PKC isoforms. Studies performed with polyclonal isoenzyme-specific antibodies, developed against various PKC isoforms, provided evidence of selective organ and tissue distribution of PKC isoforms. In rat liver predominate PKCα, δ and ϶ isoforms12. PKC isoforms α and δ are activated with TPA14. Short time TPA pre-treatment cultured hepatocytes significantly decreased insulin stimulated transport and totally abolished TNF-α effect (Figure 1). This could be a result of activation PKCα and inhibition insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 already showed by others12,13. Possible activation of PKC δ could cause serine phosphorylation of insulin receptor and on this way produce insulin resistance. Some new studies showed that activation of PKCα and δ, influences their association with insulin receptor and IRS-1 in a manner that interferes with ability of insulin to regulate these isoforms causing insulin resistance in primary cultures of mouse skeletal muscle12. Does the same mechanism is operative in primary cultures of rat hepatocytes, we presently do not know. Interestingly TNF-α effect disappeared after long time (24 h) pre-treatment period (Figure 2). Hyperinsulinaemia also, imposed on normal rats, appears to have a dual effect, first stimulating insulin signaling after that chronically decreasing it in liver and muscle. The underlying mechanism of these differential effects may be related to the ability of hyperinsulinaemia to increase IRS-1/2 serine phosphorylation10. In light of these results chronic effects of TNF-α have a completely deferent meaning. Our results indicated that TNF-α could act more specifically in hepatocytes interfering with insulin signaling and on this way modulate insulin action and possibly chronically cause insulin resistance. Other possible mediators of TNF-α induced insulin resistance in whole animal include circulating free fatty acids and leptin16. We already showed that fatty acids induced insulin resistance in the acute and reversible fashion in cultured hepatocytes17. Inasmuch as TNF-α has been shown acutely to increase lipolysis, free fatty acids could be main factor acutely responsible for the development of insulin resistance in the liver.

Acknowledgements

We thank Mr. Boris Tomasević for his expert technical assistance. This study was supported by the grant No. 0065002 from the Croatian Ministry of Science and Technology and by financial support by Zagreb Institute of Public Health.

References


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UČINCI TUMOR NEKROTIČNOG FAKTORA-α NA INZULINOM STIMULIRANI TRANSPORT AMINO KISELINA U KULTIVIRANIM HEPATOCITIMA ŠTAKORA

S A Ž E T A K

Smatra se da tumor nekrotični faktor (TNF-α) igra ključnu ulogu u patogenezi inzulinske rezistencije. On može djelovati direktno ili indirektno na jetru. Cilj istraživanja je otkriti direktna kratkoročna (4 h) i dugoročne (24 h) učinke TNF-α na transport aminokiselina u kulturi štakorskih hepatocita i moguću ulogu protein kinaze C (PKC) na inzulin signalni put i inzulinsku rezistenciju. Hepatociti su izolirani pomoću kolagenaze i kultivirani 24 sata u M-199 mediju bez dodatka seruma. Inzulin je 55% povećao bazalni transport α-amino izomasačne kiseline (AIB). TNF-α kratkoročno nije promijenio bazalni transport AIB-a, ali je signifikantno (25%) povećao inzulinom stimuliran transport. Kratkoročno djelovanje TNF-α poništeno je djelovanjem forbolnih estera. Ovi rezultati ukazuju da je aktivacija PKC važna u prijenosu inzulinskog signala i u djelovanju TNF-α. TNF-α djelujući direktno na hepatocite ne izaziva inzulinsku rezistenciju.