Effects of Inhibitors of Key Enzymes of Sphingolipid Metabolism on Insulin-Induced Glucose Uptake and Glycogen Synthesis in Liver Cells of Old Rats

N. A. Babenko* and V. S. Kharchenko

Department of Physiology of Ontogenesis, Institute of Biology, Kharkov Karazin National University, pl. Svobody 4, 61077 Kharkov, Ukraine; E-mail: babenko@univer.kharkov.ua; kharchenko_vitalina@meta.ua

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Abstract—Sphingolipids play an important role in the development of insulin resistance. Ceramides are the most potent inhibitors of insulin signal transduction. Ceramides are generated in response to stress stimuli and in old age. In this work, we studied the possible contribution of different pathways of sphingolipid metabolism in age-dependent insulin resistance development in liver cells. Inhibition of key enzymes of sphingolipid synthesis (serine palmitoyl transferase, ceramide synthase) and degradation (neutral and acidic SMases) by means of specific inhibitors (myriocin, fumonisin B1, imipramine, and GW4869) was followed with the reduction of ceramide level and partly improved insulin regulation of glucose metabolism in “old” hepatocytes. Imipramine and GW4869 decreased significantly the acidic and neutral SMase activities, respectively. Treatment of “old” cells with myriocin or fumonisin B1 reduced the elevated in old age ceramide and SM synthesis. Ceramide and SM levels and glucose metabolism regulation by insulin could be improved with concerted action of all tested inhibitors of sphingolipid turnover on hepatocytes. The data demonstrate that not only newly synthesized ceramide and SM but also neutral and acidic SMase-dependent ceramide accumulation plays an important role in development of age-dependent insulin resistance.

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Sphingolipids are a class of biologically active molecules involved in regulating physiological processes such as cell growth, apoptosis, autophagy, angiogenesis, inflammation, and neurodegeneration. Sphingolipids play an important role in the development of resistance of cell targets to insulin. Modulation of sphingolipid content in tissues of insulin action targets under in vivo conditions in experiments on cultured cells is usually accompanied by a change in their sensitivity to the action of the hormone. The most potent inhibitors of various parts in insulin signal transduction are ceramides and gangliosides. Insulin resistance induced by a high calorie diet or lipid infusion is accompanied by accumulation in the liver and muscle tissue of not only the precursor of sphingolipid synthesis de novo, palmitic acid, but also ceramide [1, 2]. In contrast, inhibitors myriocin and cycloserine of a key enzyme of sphingolipid synthesis, serine palmitoyl CoA transferase (SPT), prevent the accumulation of ceramide and normalize glucose homeostasis in the body. Using ceramide analogs easily penetrating into cells, it was shown that in muscle tissue ceramides inhibit an important enzyme of insulin signaling, protein kinase B (Akt/ PKB; EC 2.7.11.1), by suppressing the translocation of the enzyme into plasma membranes and/or dephosphorylation of protein kinase by protein phosphatase P2 (EC 3.1.3.16), which is a target for ceramide in various cells [3, 4]. The important role of ceramide in disturbance of phospholipase D (PLD)-dependent insulin signaling in liver cells of old rats has been established [5]. Suppression of sphingolipid synthesis de novo using myriocin compensates the age-related dysregulation of PLD activity by insulin (EC 3.1.4.4) but does not restore the

Abbreviations: Akt/PKB, protein kinase B; GLUT, glucose transporter; IL, interleukin; mTOR, mammalian target of rapamycin; PKC, protein kinase C; PLD, phospholipase D; SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine palmitoyl CoA transferase; TNF-α, tumor necrosis factor α. * To whom correspondence should be addressed.
level of ceramide and induction of glucose metabolism by insulin in liver cells of old rats to the level of young animals [5].

Exogenous gangliosides GM3 inhibit phosphorylation of both insulin receptor tyrosine and insulin receptor substrate 1 (IRS-1) in 3T3 L1 adipocytes [6]. Various inhibitors of glucosylceramide synthesis [7, 8] as well as knockout of enzymes [9] in muscle and liver cells enhance insulin-stimulated phosphorylation of the insulin receptor, protein kinase Akt/PKB, and mTOR (mammalian target of rapamycin). At the same time, disturbance of synthesis and lowering of content of sphingomyelin (SM) but not GM3 gangliosides in liver, muscles, adipose tissue, and plasma membranes of liver cells of mice, characterized by deficiency of SM-synthase 2 (EC 2.7.8.27) or of the second SPT subunit (EC 2.3.1.50), is an important cause of increasing insulin sensitivity of tissues [10]. It is important that the ceramide content increases in liver and muscle tissue and is not changed in plasma membranes of liver cells in SM-synthase-deficient animals and is significantly reduced in membranes of SPT-knockout mice. In view of these data and the results of other studies [11, 12], one can suggest that ceramides and gangliosides are not the only mediators in the development of insulin resistance in target tissues.

Elevation of SM levels in the plasma membranes of adipocytes and erythrocytes precedes the development of insulin resistance and of hyperinsulinemia in obese patients [13]. SM-synthase 2 deficit and disturbance of SM synthesis in knockout animals prevent induction of obesity and insulin resistance under the influence of a high fat diet [14]. Suppression of SM-synthase expression by siRNA also decreases in HepG2 cells the content of large lipid droplets and triacylglycerol in liver of leptin-deficient ob/ob mice. SM-synthase 2 is localized in lipid microdomains and is partially associated with the transporter of fatty acids CD36/FAT and with caveolin 1. Since SM-synthase 2 modulates SM content in lipid platforms, the authors concluded that it is conformational changes in membranes that mediate participation of the enzyme in regulation of obesity and type 2 diabetes. Given that SM is a component of lipid rafts, which play an important role in the compartmentalization of insulin signaling, one can propose that changes in SM content and in the ordering of the lipid bilayer of plasma membranes are an important cause of disturbance of insulin signal transduction.

Unresolved questions remain regarding what kind of sphingolipids and what kind of links in sphingolipids metabolism play a key role in the development of insulin-resistance in liver cells in old age. Therefore, the purpose of the present work was to study the effect of specific inhibitors of enzymes involved in SM and ceramide synthesis and degradation on the ability of old liver cells to provide adequate response to insulin.

MATERIALS AND METHODS

Investigations were carried out with 3-month-old (young) and 24-month-old (old) male Wistar rats. Hepatocytes were isolated by the method of Petrenko et al. [15]. Cell viability was assessed using trypan blue. Cell survival was 90-96%. Freshly isolated hepatocytes were resuspended in Eagle’s medium (Institute of Polio-myelitis and Viral Encephalitis, Russia), containing 10% fetal serum (BioloT, Russia), 20 mM Heps, penicillin (61 mg/liter), and streptomycin (100 mg/liter), up to 4×10^7 cells/ml and incubated for 3 h at 37°C. After incubation, the hepatocytes were washed with Krebs–Henseleit buffer with 0.1% BSA (Sigma, USA) and diluted prior to experiments in the same buffer. To inhibit ceramide synthesis de novo, hepatocytes of 24-month-old rats were incubated for 2 h at 37°C in the presence of 5 μM myriocin (Sigma) or 1 μM fumonisin B1 (Sigma). Ceramide synthesis was studied in the presence of [14C]palmitic acid (1 μCi/ml, 56 mCi/mmol; Amersham, GE Health Care, UK). To inhibit acidic and neutral SMases, hepatocytes of 24-month-old rats were incubated for 2 h at 37°C in the presence of 50 μM imipramine (Sigma) or 5 μM GW4869 (Sigma-Aldrich, Germany), respectively. In separate cases, the cells were incubated in the presence of both inhibitors of synthesis and degradation of sphingolipids.

To determine activity of SMases (EC 3.1.4.12), hepatocytes were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM MgCl2, and 0.65% Triton X-100 (for subsequent determination of the activity of neutral SMase) or in buffer containing 50 mM CH3COONa, pH 5.0, 0.65% Triton X-100 (for subsequent determination of the activity of acidic SMase). The reaction mixture contained 1.5 mg protein and 0.74 nmol [methyl-14C-choline]SM (52 mCi/mmol; PerkinElmer, USA) in a final volume of 200 μl. Samples were incubated for 1 h, and the reaction was stopped with 1.5 ml of CHCl₃–CH₃OH (1 : 2 v/v) mixture followed by addition of 1 ml of CHCl₃ and 1 ml of H₂O. The mixture was centrifuged for 5 min at 3000 rpm. After phase separation, aliquots of the upper and lower phases containing [14C]phosphorylcholine and [14C]SM, respectively, were used for determination of radioactivity. Enzyme activity was expressed as nmol of product or substrate per mg of protein per hour.

Non-[14C]-labeled hepatocytes were used to study insulin-induced (mono-component porcine insulin; Indar, Ukraine) uptake of 2-D-[3H]glucose (0.5 μCi/ml) and incorporation of D-[U-14C]glucose (0.1 μCi/ml) into glycogen by the method of Brutman-Barazani et al. [16]. Hepatocytes were preincubated in the presence or absence of sphingolipid metabolism inhibitors for 2 h and then washed free from the additions by HBS buffer (HEPES-buffered saline) containing 20 mM HEPES. Then hepatocytes were incubated in the same buffer for