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Ceramides Inhibit Phospholipase D-Dependent Insulin Signaling in Liver Cells of Old Rats

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Abstract—Ceramides are a novel class of biologically active molecules involved in the regulation of different signaling pathways. Ceramide is involved in regulation of the phospholipase D (PLD) activity and development of cell resistance to insulin. In this work, we have studied age-related features of insulin regulation of PLD activity and glucose metabolism in intact cells and modeled their resistance to insulin by exogenous ceramide and palmitic acid. Contents of ceramides and of free fatty acids (FFA) are found to increase with age, as well as on incubation of liver cells of young rats in the presence of the ceramide precursor palmitic acid. Under these conditions, the ability of insulin to activate PLD, the cell uptake of glucose, and glycogen synthesis sharply decreased. On incubation of hepatocytes of young animals in the presence of exogenous C2-ceramide, the contents of endogenous ceramides increased but not the contents of FFAs and of neutral lipids. These events were accompanied by suppression of the insulin-induced production of phosphatidylethanol (a result of ethanol transphosphatidylation by PLD), glucose uptake, and glycogen synthesis. Incubation of insulin-resistant liver cells of young rats and also of hepatocytes of old rats in the presence of myriocin (an inhibitor of the *de novo* synthesis of ceramide) was associated with a decrease in ceramide content in the cells and an increase in the cell sensitivity to insulin. The findings indicate an important role of ceramide in disturbance of insulin signaling due to inhibition of the PLD-dependent link in the liver cells of old animals.

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Key words: ceramide, myriocin, phospholipase D, hepatocytes, insulin resistance, aging

Ceramide, an important regulator of cell growth and apoptosis, is a product of hydrolysis of sphingomyelin by different sphingomyelinases or is synthesized *de novo*. Sphingolipid metabolism in rat liver increases with age [1, 2]. On aging, the activity of sphingomyelinases increases, and ceramide is accumulated in fibroblasts [3], liver, and hippocampus of the rats [4, 5]. Ceramide regulates age-related biochemical and genetic processes [6]. An increased content of ceramide in cells suppresses signaling pathways responsible for transmission of hormonal signals, including those of insulin, and this can cause a resistance to insulin [7]. In the liver and muscles of insulin-resistant rats the ceramide level is significantly higher than in the tissues of normal animals [8].

Abbreviations: DAG, diacylglycerol; FFA, free fatty acids; GLUT, glucose transporter; PC, phosphatidylcholine; PET, phosphatidylethanol; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLD, phospholipase D; 16:0, palmitic acid.

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Phospholipase D (PLD) is known to be a target of insulin. Phosphatidylcholine (PC)-specific PLD is activated by insulin in plasma membranes of rat adipocytes, BC3H-1 myocytes, and rat hepatocytes [9]. In HEK 293 cells and rat hepatocytes, insulin stimulates protein kinase C (PKC)- and PLCγ-dependent activity of PLD [10, 11]. In rat adipocytes the insulin-induced activation of phosphatidylinositol-3-kinase leads to production of polyphosphoinositides in the plasma membrane with subsequent translocation of Rho, activation of PLD [12], and increase in glucose transport. Insulin is shown to activate PLD in the cells with translocated glucose carrier GLUT-4 [13]. Along with atypical PKC forms ζ and λ and protein kinase B, PLD is involved in regulation of the key stage of glucose transport, in particular, the translocation of glucose carriers from the endoplasmic reticulum into the plasma membrane [13]. There are data on the identity of molecular mechanisms that control the GLUT-2 and GLUT-4 translocation, because the signaling pathway PI3K/PLD/PKC is

involved in the regulation of traffic of these carriers [14, 15].

Disorders in functioning of the signaling pathway mediated by PLD/DAG/PKC have been observed in aging fibroblasts, and this is supposed to be associated with the accumulation of ceramide [16]. As shown in work [17], ceramide inhibits PLD in competition with phosphoinositide PtdIns(4,5)P_2 for the catalytic site of the enzyme. Ceramide is also shown to partially inhibit translocation of PLD activators ARF protein and PKC, which results in a decrease in the PLD activity [18]. Moreover, the ceramide-caused destruction of lipid raft structure correlates with inhibition of PLD [19].

At present, age-related mechanisms of development of insulin resistance of liver cells are still unclear, as well as the role of PLD and ceramides in this process. The purpose of the present work was to study age-related features of the PLD-dependent link of the insulin signal transmission in liver cells, to elucidate the role of ceramide in the disturbance of this transmission, and to search for a possibility of correcting the state of insulin resistance in old cells by modifying sphingolipid metabolism.

MATERIALS AND METHODS

Studies were performed on 3- and 24-month-old male Wistar rats. Before opening the abdominal cavity, the animals were anesthetized with diethylether. Hepatocytes were isolate as described in work [20]. The cell viability was estimated using Trypan Blue. The cell survival was 90-96%. Freshly isolated hepatocytes were resuspended in Eagle medium (Institute of Poliomyelitis and Viral Encephalitis, Russia) supplemented with 10% fetal serum (BioloT, Russia), 20 mM HEPES, penicillin (61 mg/liter), streptomycin (100 mg/liter), ~$4 \cdot 10^7$ cell/ml, and incubated for 3 h at 37°C in the presence of C2-ceramide (D-erythro-*N*-acetylsphingosine) (Amersham, England) (5 μg per ml of the medium), or 0.75 mM palmitic acid (16:0) (Sigma, USA), or the control mixture (dodecane–ethanol, 49 : 1, in the equivalent volume). Before addition into the medium, a complex of 16:0 with BSA (Sigma) was prepared as described in [21]. To inhibit *de novo* ceramide synthesis, the incubation medium for 3-month-old rat hepatocytes was supplemented with 5 μM myriocin (Sigma) 30 min before the addition of 16:0 or C2-ceramide. The total time of incubation with the inhibitor was 3 h. Hepatocytes of 24-month-old rats were also incubated for 3 h in the presence of myriocin at 37°C. After the incubation, the hepatocytes were washed in Krebs–Henseleit buffer with 0.1% BSA and before experiment were diluted in the same buffer. The hepatocyte concentration was ~$2 \cdot 10^7$ cell/ml. Then the insulin-induced uptake of [^{3}H]D-glucose (0.5 μCi/ml) by the cells and inclusion of [U^{14}C]D-glucose (0.1 μCi/ml) into glycogen were determined as described in work [22]. Radioactivity of [^{3}H]D-glucose and [^{14}C]glycogen was determined using a β-radioactivity counter. Insulin was from Indar (Ukraine).

The PLD activity was determined using an approach based on formation of phosphatidylethanol (PET), i.e. of a phospholipid, which is produced only by PLD as a result of transphosphatidylation in the presence of ethanol [23-26]. As discriminated from phosphatidic acid, PET is metabolized very slowly and therefore can be used as an indicator of PLD activation in stimulated cells. To determine the PLD activity, the hepatocyte suspension was incubated in the presence of [^{14}C]palmitic acid (16:0) (0.25 μCi/ml) (Amersham) for 90 min, then the cells were washed in Krebs–Henseleit buffer with c 0.1% BSA and diluted in the same buffer to the cell concentration of $2 \cdot 10^7$ cell/ml. Before addition of insulin into the incubation medium, the cells were preincubated for 10 min with 300 mM ethanol, and then 10 nM insulin or 0.9% NaCl (as a control for insulin) was added into the medium and the reaction was stopped after 5 or 30 min. Lipids were extracted as described in [27]. Individual lipids were separated by TLC on Sorbfil plates (Sorbpolymer, Russia) in solvent systems as followed: $CH_3COOCH_2CH_3$–isooctane–CH_3COOH–H_2O (130 : 20 : 30 : 100 v/v) for PET; hexane–diethyl ether–glacial CH_3COOH (73 : 25 : 2 v/v) for free fatty acids (FFA). Phospho- and sphingolipids were separated in the systems: $CH_3CH_2OCH_2CH_3$ (system 1) and $CHCl_3$–CH_3OH–H_2O (40 : 10 : 1 v/v) (system 2). Lipid spots were developed with iodine vapor and identified by comparing with standards. Contents of neutral lipids in the specimens were determined as described in [28], and contents of phospholipids were determined as described in [29]. To quantitatively determine ceramide contents in cells, the lipid spots were transferred into tubes and eluted with chloroform–methanol mixture (1 : 1 v/v) with subsequent elution by methanol. The pooled eluates were evaporated under vacuum and hydrolyzed in 0.5 M HCl solution in methanol at 65°C for 15 h. The weight of ceramides was determined by the release of long-chained compounds during the lipid hydrolysis [30]. The results are presented as mean value ± standard error of the mean. Two groups were compared using Student's *t*-test, the data obtained upon multiple influences being compared by multifactorial analysis of variance.

RESULTS

A short-term stimulation by insulin of hepatocytes of young rats activated PLD. This was directly confirmed by insulin-induced generation of [^{14}C]PET as a result of the PLD-specific transphosphatidylation of ethanol (Fig. 1a). Insulin also decreased in the cells the level of a PLD substrate PC (Fig. 1a) and increased the uptake of

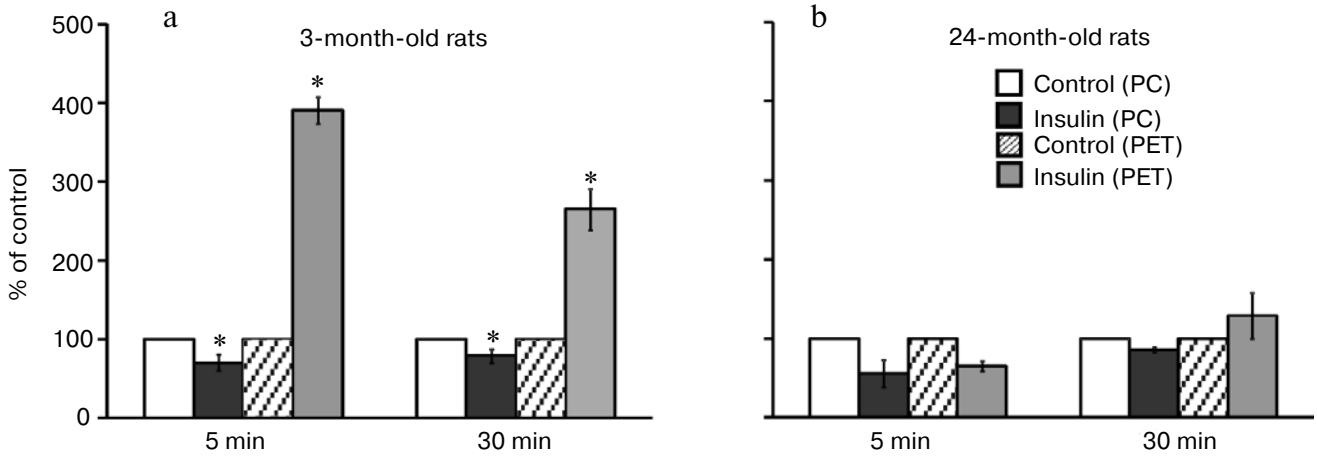

Fig. 1. Effect of insulin on the phospholipase D activity in isolated hepatocytes from rats of different age: a) 3-month-old rats ($n = 7$); b) 24-month-old rats ($n = 6$). * Significant with respect to control for insulin (0.9% NaCl), $p < 0.05$.

[^{14}C]glucose and glycogen synthesis by the hepatocytes of 3-month-old rats (Fig. 2).

However, insulin did not activate PLD in the liver of 24-month-old rats (Fig. 1b), and the ability of insulin to induce glucose uptake and glycogen synthesis was sharply decreased (Fig. 2) on the background of an increased accumulation of FFAs and ceramides as compared to the 3-month-old rats. The FFA content in hepatocytes of 3-month-old rats was 2.6 ± 0.24 nmol/10^6 cells, whereas in 24-month-old rats it was 3.71 ± 0.28 nmol/10^6 cells ($p < 0.05$). The ceramide level in 3-month-old rats was 2.62 ± 0.13 nmol/10^6 cells, and in 24-month-old rats it was 31.5 ± 1.49 nmol/10^6 cells ($p < 0.05$). These findings are in agreement with results of previous studies [4].

Then we wanted to elucidate whether the accumulation of newly synthesized long-chained ceramides in old cells could cause development of their resistance to insulin. Before addition of insulin into the incubation medium, hepatocytes of 24-month-old rats were pretreated with myriocin, which was an inhibitor of serine palmitoyl transferase – a key enzyme of sphingolipid synthesis. The preincubation with myriocin decreased by 59% the level of newly synthesized ceramides in the liver cells of 24-month-old rats as compared to intact hepatocytes of old rats. The ceramide content in hepatocytes of 24-month-old rats (control) was 31.5 ± 1.49 nmol/10^6 cells; in the cells preincubated with myriocin the ceramide content was 15.3 ± 1.05 nmol/10^6 cells ($p < 0.05$).

Along with the myriocin-caused decrease in the ceramide content in the liver cells of old animals in response to insulin, the PLD activity and glucose metabolism were stimulated (table).

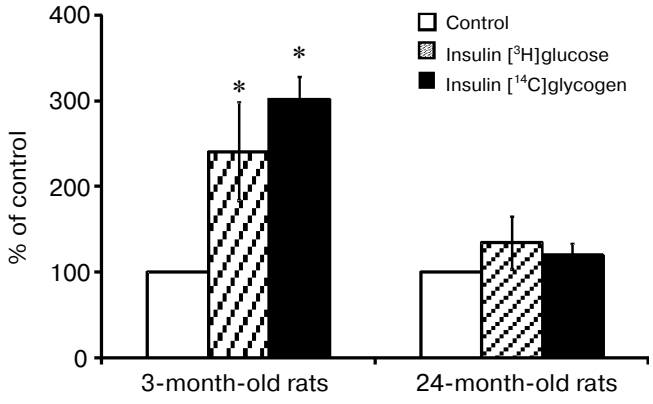

Fig. 2. Effect of insulin on glucose uptake and glycogen synthesis in isolated hepatocytes from 3- and 24-month-old rats ($n = 7$). * Significant with respect to control for insulin (0.9% NaCl), $p < 0.05$.

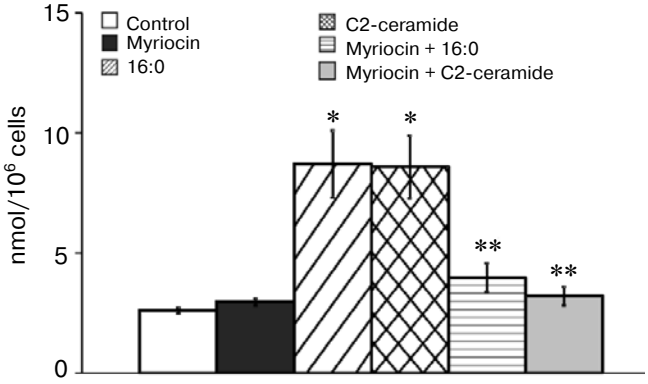

Fig. 3. Effect of palmitic acid, exogenous C2-ceramide, and myriocin on ceramide content in liver cells of 3-month-old rats ($n = 7$-10). * Significant with respect to control, $p < 0.05$. ** Significant with respect to 16:0/C2-ceramide, $p < 0.05$.

Influence of myriocin on insulin-induced PLD activation, glucose uptake, and glycogen synthesis in hepatocytes of 24-month-old-rats under different experimental conditions

Insulin induced	Radioactivity, cpm/10^6 cells		
	control	insulin	insulin + myriocin
Generation of [^{14}C]PET	15.7 ± 0.25	14. 4 ± 0.5	21.3 ± 0.8*, **
Uptake of [^{3}H]glucose	12.7 ± 2.9	13.0 ± 6.1	13.9 ± 3.4*
Synthesis of [^{14}C]glycogen	106.6 ± 1.3	95.9 ± 1.4*	116.1 ± 2.3*

* Significant with respect to control, $p < 0.05$.
** Significant with respect to insulin, $p < 0.05$.

Modeling the insulin resistance of cultured hepatocytes of young rats in the presence of an increased concentration of 16:0 or exogenous C2-ceramide increased in these cells the content of endogenous ceramides (Fig. 3). Note that the ceramide levels in the cells incubated in the presence of 16:0 and C2-ceramide were virtually the same.

Exogenous 16:0 and C2-ceramide suppressed the insulin-induced generation of [^{14}C]PET (Fig. 4), and this indicated that the insulin-induced activation of PLD was inhibited.

Both 16:0 and exogenous C2-ceramide prevented the insulin-induced glucose uptake and glycogen synthesis in hepatocytes of young animals (Fig. 5). These data indicated the development of insulin resistance in hepatocytes of young rats incubated in the presence of 16:0 and C2-ceramide.

To elucidate whether the newly synthesized ceramides could cause disorders in insulin signaling, hepatocytes were pretreated with myriocin before the introduction of 16:0 or C2-ceramide into the incubation medium. In hepatocytes of 3-month-old rats preincubated with myriocin and 16:0 (or C2-ceramide), the ceramide content was decreased as compared to that in hepatocytes incubated in the presence only of 16:0 or C2-ceramide (Fig. 3). Myriocin prevented the effect of 16:0 or C2-ceramide on the PLD activity (Fig. 4) and increased but failed to completely recover the stimulatory

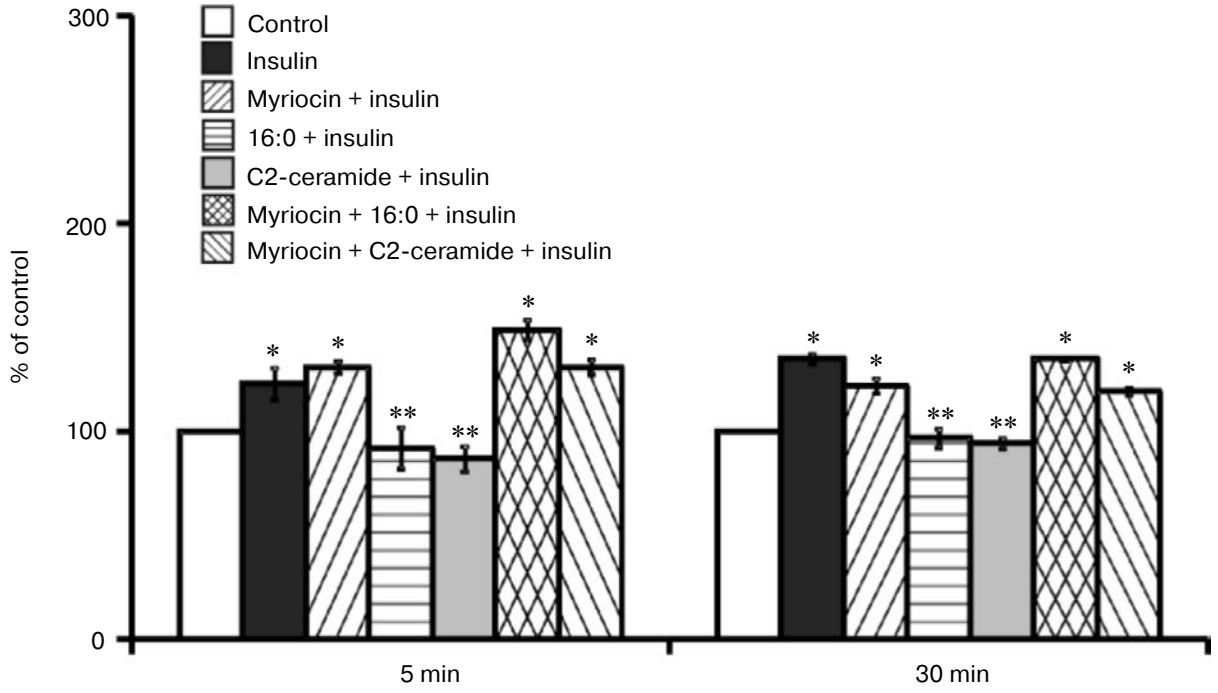

Fig. 4. Effect of palmitic acid, exogenous C2-ceramide, and myriocin on insulin-induced activation of phospholipase D in hepatocytes of 3-month-old rats (n = 7-10). * Significant with respect to control (0.9% NaCl), $p < 0.05$. ** Significant with respect to insulin, $p < 0.05$.

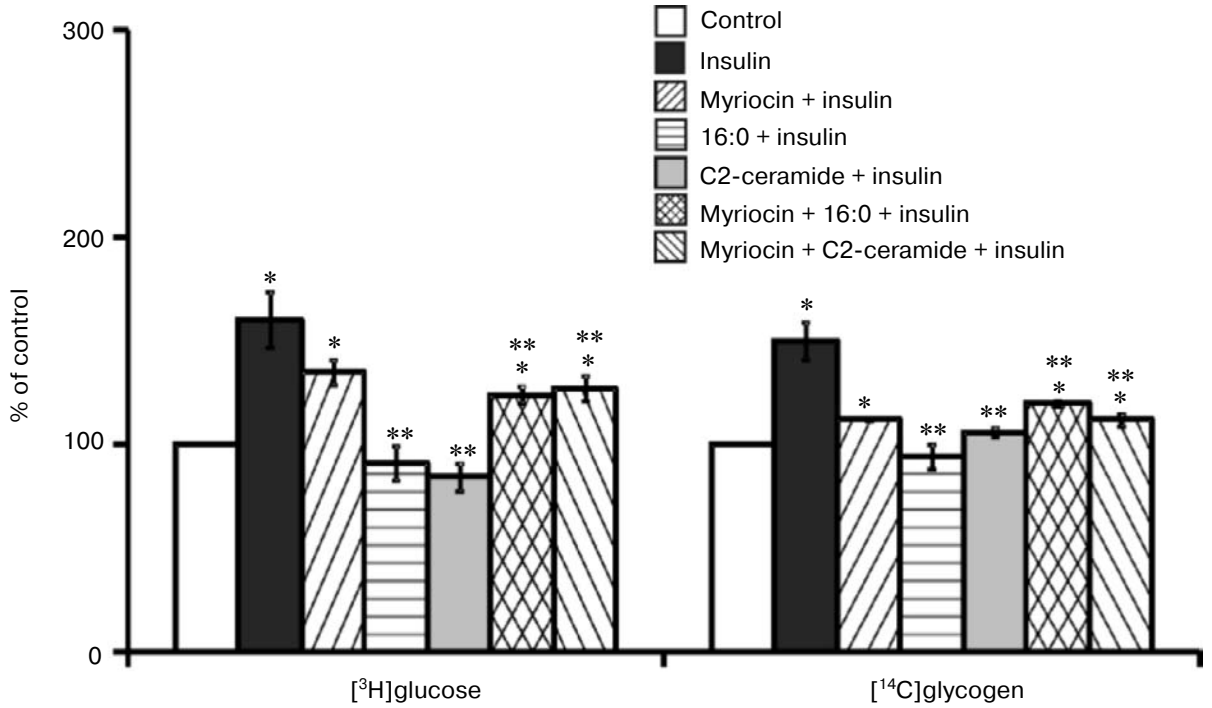

Fig. 5. Effect of exogenous palmitic acid, C2-ceramide, and myriocin on the insulin-induced induction of glucose uptake and glycogen synthesis in hepatocytes of 3-month-old rats (n = 7-10). * Significant with respect to control (0.9% NaCl), $p < 0.05$. ** Significant with respect to insulin, $p < 0.05$.

effect of insulin on glucose metabolism in young cells with induced resistance to insulin (Fig. 5).

DISCUSSION

Induction by ceramide of cell resistance to insulin is characteristic for all classic target tissues of insulin, i.e. for skeletal muscles, fatty tissue, and liver [31]. An increase in production of endogenous ceramide under the influence of saturated fatty acids in the diet contributes to the development of cell resistance to insulin and type 2 diabetes [32, 33]. Ceramide is known to inhibit the insulin signaling in insulin-sensitive cells through inhibition of Akt/PKB due to induction of dephosphorylation of the protein kinase and prevention of its translocation into the plasma membrane and the resulting suppression of the glycogen synthesis. However, an important ceramide target in cells is PLD, which positively modifies the glucose transport in the insulin-stimulated cells [34]. Ceramide was earlier shown to inhibit PLD [35-37]. The aging of fibroblasts in culture was shown to be accompanied by accumulation of ceramides and a decrease in the PLD activity, and it was concluded that accumulation of ceramides in old cells could be an important cause suppressing PLD. An increase in contents of saturated fatty acids, which are precursors of sphingolipid synthesis, and ceramides has been observed in old age. The ability of hepatocytes isolated from old animals to adequately respond to insulin is sharply decreased as compared to such ability of the cells from young rats. In hepatocytes of old rats, insulin virtually failed to influence the PLD-dependent link of insulin signaling on the background of a pronounced accumulation of ceramide, which is an endogenous inhibitor of PLD. Disorders in PLD activation by insulin lead to a decrease in phosphatidic acid production and, as a result, to inhibition of the binding and fusion of vesicles containing glucose carriers with the plasma membrane [38]. Ceramide can influence molecules responsible for activating PLD [18, 19] and also cofactors that are necessary for the PLD activation [17]; moreover, ceramide can inhibit PLD on the transcription level [39]. Incubation of myoblasts in the presence of C6-ceramide decreased PLD1 expression in vasopressin-stimulated cells [40]. However, the addition into the culture medium of ceramide synthesis inhibitors fumonisin B1 or myriocin increased PLD1 expression and PLD activity. It was concluded that ceramide acting on the transcription level could selectively regulate the PLD1 activity and that the modifying action of sphingolipid synthesis inhibitors on the PLD activity should be partially mediated through changes in PLD1 expression. Based on these data and also on results of the present work, it was suggested that inhibition of PLD under the influence of endogenous ceramides could be a significant cause of the age-related development of cell resistance to insulin.

This hypothesis is also supported by data of the present work that the inhibition of ceramides synthesis and the decrease in their level in the liver cells of old animals by myriocin (a specific inhibitor of serine palmitoyl transferase) increased the sensitivity of hepatocytes to insulin. The myriocin-induced decrease in the ceramide level in the old cells was accompanied by the insulin-induced activation of PLD and of glucose metabolism. These findings indicate that the increase in the level of the newly synthesized ceramide in the cells is a significant cause of decrease in cell sensitivity to insulin in old age.

During the development of insulin resistance, ceramide is mainly synthesized *de novo* [41]. Inhibition of key enzymes of sphingolipid biosynthesis, such as serine palmitoyl transferase, ceramide synthase, and dihydroceramide desaturase, increases the cell sensitivity to insulin [33, 41, 42]. On inhibition of the *de novo* ceramide synthesis by myriocin in insulin-resistant mice suffering obesity, the treatment with myriocin resulted in recovery of sensitivity to insulin of target tissues [7, 33]. Based on the literature data and results of the present work, it is supposed that the suppression of ceramide synthesis in liver cells should allow us to increase their sensitivity to insulin and thus to prevent the development of resistance to insulin in old age.

To test this hypothesis and also to elucidate the role of PLD in insulin signaling, experiments were performed on hepatocytes isolated from young animals with the induced insulin resistance. Cultivation of these liver cells in the presence of exogenous C2-ceramide easily penetrating into the cells or in the presence of 16:0, which is a precursor of sphingolipid synthesis, resulted in accumulation in the cells of endogenous ceramides. The level of endogenous ceramides in the presence of C2-ceramide can increase in the cells due to activation of the deacylation–reacylation reaction [43]. However, in cultured keratinocytes both C2- and C6-ceramide increased the level of newly synthesized ceramides due to activation of the key enzyme of sphingolipid synthesis – serine palmitoyl transferase [44]. Introduction into the medium of myriocin, and inhibitor of this enzyme, prevented the induction by short-chained ceramides of endogenous ceramide accumulation. In the present work it was also shown that myriocin normalized the content of endogenous ceramides in hepatocytes of old rats and in young hepatocytes cultured in the presence of both C2-ceramide and 16:0, and this confirmed that activation of *de novo* synthesis of ceramide caused its accumulation in the liver cells. Endogenous C2-ceramide and 16:0 completely suppressed the activating effect of insulin on both PLD and glucose metabolism in hepatocytes of young animals. Due to their surface activity, 16:0 and C2-ceramide can influence the availability of the substrate for the enzyme and lead to sorption of PLD on micelles and lowering the effective enzyme concentration in the transphosphatidylation sites. However, the normalization of the ceramide level by myriocin completely recovered the activating effect of insulin on PLD in the insulin-resistant cells of young rats, and this confirmed that 16:0 and C2-ceramide mainly acted on PLD in the insulin-stimulated cells due to increase in the content of newly synthesized ceramides. However, myriocin increased but failed to recover the effect of insulin on glucose transport and glycogen synthesis in insulin-resistant hepatocytes. These findings suggest that newly synthesized ceramides inhibit the PLD-dependent link of the insulin signaling and do not influence other pathways of insulin signal transmission.

Thus, it was established in the present work that liver cell sensitivity to insulin is sharply decreased in old age. An important role in development of the age-related resistance of hepatocytes to insulin is played by ceramides, whose level in cells increases with aging. Activation of *de novo* synthesis of ceramides contributes to a decrease in the cell sensitivity to insulin. In insulin-stimulated liver cells PLD is a target for ceramides. Inhibition of the PLD stimulation by insulin under the influence of ceramide is accompanied by a decrease in insulin-activated glucose metabolism. Resistance to insulin arising in the old age and induced in young cells is partially reversible, and this indicates that the ceramide-caused suppression of the PLD stimulation by insulin is a significant but not the only cause of disorders in the regulation of glucose metabolism by insulin.

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