Ceramide is involved in the pathogenesis of insulin resistance in skeletal muscles of humans and rodents. However, there are conflicting reports in the literature on the effect of thiazolidinediones (a new class of insulin sensitizing drugs) on skeletal muscle ceramide content. Therefore, the aim of our study was to examine the effect of pioglitazone on the level of ceramide and its metabolites and on the activity of the key enzymes of ceramide metabolism in different skeletal muscle types of the rat. The experiments were carried out on rats fed either a standard chow or a high-fat diet for 21 days. Each group was divided into two subgroups: control and treated with pioglitazone for 14 days. High-fat diet increased the content of ceramide in the soleus and in the red section of the gastrocnemius, but not in the white section of the latter. The activity of neutral Mg$^{2+}$-dependent sphingomyelinase and acid sphingomyelinase was simultaneously reduced in all examined muscles. Administration of pioglitazone decreased ceramide level in the soleus and in the red section of the gastrocnemius in rats fed either diet. This effect could not be attributed to decreased rate of ceramide formation from sphingomyelin or to its augmented deacylation to sphingosine. Pioglitazone treatment reduced the concentration of plasma free fatty acids in rats fed on either diet. Therefore, we conclude that the drug decreased the muscle content of ceramide by reducing its \textit{de novo} synthesis. The results of our study indicate that reduction in ceramide level may be one of the mechanisms by which pioglitazone improves skeletal muscle insulin sensitivity.

\textbf{Key words:} \textit{PPAR, pioglitazone, ceramide, sphingomyelinase, ceramidase, skeletal muscle, high-fat diet}

\textbf{INTRODUCTION}

Ceramide (CER) is the second messenger in so-called sphingomyelin signaling pathway (1, 2) and is involved in the regulation of various cellular processes such as proliferation, differentiation, apoptosis and inflammation (3). Ceramide is also an
important mediator of lipotoxicity and insulin resistance (4). The major route of acute CER formation is hydrolysis of sphingomyelin (SM) by the action of the enzyme sphingomyelinase. Ceramide is also synthesized de novo in Golgi apparatus. The first step in this pathway is condensation of serine and palmitoyl-CoA catalyzed by the enzyme serine palmitoyltransferase. CER is deacylated by the enzyme ceramidase. Sphingosine, the product of this reaction, can be further phosphorylated to form sphingosine-1-phosphate (S1P). Both compounds are bioactive sphingolipids (3). All the mediators and key enzymes of sphingomyelin signaling pathway were shown to be present in rat and human skeletal muscles (5 - 9).

Several factors were found to affect skeletal muscle CER metabolism. Dobrzyń and Górski (5) reported that prolonged exercise decreased the content of ceramide and the activity of neutral Mg$^{2+}$-dependent sphingomyelinase (N-SMase) in rat skeletal muscles. Moreover, it was shown that acute exercise induced accumulation of ceramide metabolites: sphingosine and sphinganine in rat skeletal muscles (7). The reduction in muscle CER content was also reported in rats subjected to endurance training (10). On the other hand, in insulin resistant and diabetic humans and rodents the level of ceramide in skeletal muscles was found to be markedly increased (6, 11 - 13).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor superfamily. Three distinct PPAR isoforms termed α, δ and γ have been described, of which all are expressed in skeletal muscles (14). Pioglitazone is an insulin-sensitizing drug belonging to the thiazolidinedione class which has been proved to be effective in the treatment of Type 2 diabetes. It exerts its effect as high affinity agonist of PPARγ (15). In skeletal muscle the major effect of PPARγ activation is the increase in basal and insulin-stimulated glucose uptake, which is the consequence of increased GLUT1 expression and translocation of GLUT4 to the plasma membrane, respectively (16, 17).

There are conflicting reports in the literature on the effect of thiazolidinediones on skeletal muscle ceramide content. Lessard et al. (18) showed that administration of rosiglitazone markedly increased the content of CER in the soleus muscle of obese Zucker rats. On the contrary, treatment with troglitazone reduced skeletal muscle ceramide level in mice (19). However, the above-mentioned studies did not address the mechanism of this phenomenon. Therefore, the aim of our study was to examine the effects of PPARγ activation on the content of CER and its metabolites and on the activity of key enzymes of ceramide metabolism in different skeletal muscle types of the rat.

**MATERIALS AND METHODS**

*Animals and study design*

The experimental protocol was approved by the Ethical Committee for Animal Experiments at the Medical University of Bialystok. Male Wistar rats (200-250 grams of body weight) were housed...
under controlled conditions (21 °C ± 2, 12 h light/12 h dark cycle) with unlimited access to water. The animals were divided into two groups: 1) fed ad libitum on a standard laboratory rat chow (Agropol, Motycz, Poland) containing 2.8% of fat by weight (n=20), 2) fed for three weeks on isocaloric high-fat diet containing 33.9% of fat by weight (n=20), prepared as described by Pascoe and Storlien (20). Each group was further divided into two subgroups: a) control (n=10) and b) treated daily for two weeks with a selective PPARγ agonist – pioglitazone (“Actos”, Lilly) in a dose of 3 mg/kg of body weight starting from the second week of the experiment (n=10). The drug was suspended in 0.5% methylcellulose and administrated by an oral gavage. The animals were anaesthetized by intraperitoneal injection of pentobarbital in a dose of 80 mg/kg of body weight. The soleus and the red (RG) and white (WG) sections of the gastrocnemius were excised and immediately freeze-clamped with aluminum tongs precooled in liquid nitrogen and then stored at -80 °C until analysis.

**Sphingomyelin and ceramide content**

The samples were pulverized in an aluminum mortar precooled in liquid nitrogen. The powder was then transferred to a tube containing methanol and 0.01% butylated hydroxytoluene (Sigma) as an antioxidant. Lipids were extracted by the method of Folch. Next, ceramide and sphingomyelin were isolated by means of thin-layer chromatography (TLC) using the methods described by Yano et al. (21) and Mahadevappa et al. (22), respectively. Further analysis was performed as described, in detail, elsewhere (5). Briefly, the gel bands corresponding to the standards were scrapped of the plates and transferred into screw-cap tubes containing pentadecanoic acid (Sigma) as an internal standard. Ceramide and sphingomyelin fatty acids were then transmethylated in the presence of 14% boron trifluoride (Sigma) in methanol at 100 °C for 90 min. The fatty acid methyl esters were analyzed by means of gas-liquid chromatography. A Hewlett-Packard 5890 Series II system equipped with a double flame ionization detector and Agilent CP-Sil 88 capillary column (100 m, internal diameter of 0.25 mm) were used. The content of ceramide and sphingomyelin is presented as the sum of individual fatty acid residues.

**The concentration of plasma free fatty acids**

Lipids were extracted from the samples as described above and the fraction of free fatty acids (FFA) was isolated by means of TLC according to Roemen and van der Vusse (23). The gel bands corresponding to the FFA standard were scrapped of the plates and transferred into fresh tubes. FFA were then transmethylated and the content of their methyl esters was determined by means of gas-liquid chromatography as previously described in detail (24).

**The content of sphingosine, sphinganine and sphingosine-1-phosphate**

The content of sphingosine, sphinganine and S1P was measured simultaneously by the method of Min et al. (25). Briefly, tissues were homogenized in a solution composed of 25 mM HCl and 1 M NaCl. Acidified methanol and internal standards (C17-sphingosine and C17-S1P, Avanti Polar Lipids) were added and the samples were ultrasonicated in ice-cold water for 1 min. Lipids were then extracted by the addition of chloroform, 1 M NaCl and 3 N NaOH. The alkaline aqueous phase containing S1P was transferred to a fresh tube. The residual S1P in the chloroform phase was reextracted twice with methanol /1 M NaCl (1:1, v/v) solution and then all the aqueous fractions were combined. The amount of S1P was determined indirectly after dephosphorylation to sphingosine with the use of alkaline phosphatase (bovine intestinal mucosa, Fluka). To improve the extraction yield of released sphingosine some chloroform was carefully placed at the bottom of the reaction tubes. The CHCl3 fractions containing free sphingosine and sphinganine or sphingosine
liberated from S1P were washed with alkaline water (pH adjusted to 10.0 with ammonium hydroxide) and then evaporated under a nitrogen stream. The dried lipid residues were redissolved in ethanol, converted to their o-phthalaldehyde derivatives and analyzed on a HPLC system (ProStar, Varian Inc.) equipped with a fluorescence detector and C18 reversed-phase column (Varian Inc. OmniSpher 5, 4.6 mm i.d. × 150 mm). The isocratic eluent composition of acetonitrile (Merck):water (9:1, v/v) and a flow rate of 1 ml/min were used.

The activity of sphingomyelinases

The activity of neutral Mg$^{2+}$-dependent and acid sphingomyelinase (N- and A-SMase, respectively) was determined as reported by Liu and Hannun (26). Briefly, the muscle homogenates were centrifuged at 1000 × g for 10 min and 50 µl of the supernatant was used for further analysis. The activity of both sphingomyelinases was measured using radiolabeled substrate, [N-methyl-$^{14}$C]-sphingomyelin (Perkin-Elmer Life Sciences). In the case of N-SMase, the reaction mixture contained 100 nmol of sphingomyelin (1154 dpm/nmol) in 100 mM Tris-HCl (pH 7.4), 5 mM MgCl$_2$, 0.1% Triton X-100 and 5 mM dithiothreitol in a final volume of 0.2 ml. In the case of A-SMase, the assay mixture contained 100 nmol of sphingomyelin (1154 dpm/nmol) in 100 mM sodium acetate (pH 5.0), 0.1% Triton X-100 and 0.1 mM EDTA. After incubation at 37 °C for 1 h the reaction was stopped by adding 1.5 ml of chloroform:methanol (2:1 v/v), followed by addition of 0.2 ml of water. A portion of the aqueous phase was transferred to scintillation vials and counted in a liquid scintillation counter for the radioactivity of the reaction product, $^{14}$C-choline phosphate.

The activity of ceramidases

The activity of alkaline (Al-CDase) and neutral (N-CDase) ceramidase was determined by the method of Nikolova-Karakashian and Merrill (27). The activity of the enzymes was measured using radiolabeled substrate, [N-palmitoyl-$^{14}$C]-sphingosine (Moravek Biochemicals). The tissue homogenates were centrifuged at 1000 × g for 10 min and 50 µl of the supernatant was used for the analysis. The reaction was started by the addition of supernatant to the tubes containing 20 µl of substrate mixture (50 nmol of ceramide – 2353 dpm/nmol, 2.5 mg Triton X-100, 1 mg Tween 20, 0.4 mg sodium cholate) and 130 µl of a reaction buffer. The reaction buffer contained 125 mM sucrose, 0.01 mM EDTA and 100 mM Tris-HCl (pH 7.2) or 125 mM HEPES (pH 8.0) for N-CDase and Al-CDase activity assay, respectively. After incubation at 37 °C for 1 h the reaction was stopped by adding 2 ml of basic Doyle’s solution (isopropanol:heptane:1 N NaOH, 40:10:1, v/v/v), 1.8 ml of heptane and 1.6 ml of water. Samples were then centrifuged and the upper phase was discarded. The lower phase was washed with 1.6 ml of heptane and then 1 ml of 1 N H$_2$SO$_4$ and 2.4 ml of heptane were added. After centrifugation, aliquots from the upper phase were transferred to scintillation vials and analyzed for the radioactivity of the reaction product, $^{14}$C-palmitate.

Protein content

Protein content was measured with BCA protein assay kit (Sigma) according to the manufacturer’s instructions. Bovine serum albumin (fatty acid free, Sigma) was used as a standard.

Statistical analysis

All data are presented as means ± SD. Statistical comparisons were made by using two-way analysis of variance followed by Newman-Keuls test. If variances were heterogeneous among groups, Dunnett’s T3 test was used instead. p<0.05 was considered statistically significant.
RESULTS

General features of the experimental animals (Table 1)

Pioglitazone treatment of the control group fed on the standard chow did not produce significant alterations in weight gain. High-fat feeding of control rats increased the weight gain, however, the difference did not reach statistical significance (p<0.07). In animals fed on the high-fat diet pioglitazone administration considerably increased the weight gain.

High-fat diet elevated the plasma FFA concentration in the control rats. Treatment with pioglitazone decreased the concentration of plasma FFA in animals fed both on the standard and on the high-fat diet by 44 and 31% respectively.

The content of sphingomyelin and ceramide (Fig. 1)

In control animals fed on the standard chow the content of sphingomyelin was highest in the soleus and lowest in the WG, with the content in RG in between. High-fat diet increased the level of sphingomyelin in all examined muscle types. Administration of pioglitazone reduced the content of sphingomyelin in the soleus and RG in rats fed either diet. In WG PPARγ activator did not affect the level of sphingomyelin in either group. In control rats fed on the standard chow the content of ceramide was higher in the soleus as compared to the RG and WG. High-fat diet caused an increase in the level of ceramide in the soleus and RG and had no effect in the WG. Treatment with pioglitazone reduced the content of CER in the soleus and RG in rats fed on either diet. The level of ceramide in the WG was not altered by the PPARγ activator, irrespectively of the diet.

The content of sphinganine, sphingosine and S1P (Fig. 2)

In control animals fed on the standard chow the content of sphinganine, sphingosine and S1P was significantly lower in the WG comparing to the soleus and RG. High-fat diet increased the content of sphinganine in the soleus and did not affect its level in other examined muscle types. In rats fed on the standard chow pioglitazone did not alter the content of sphinganine in either muscle. However, in high-fat fed animals administration of PPARγ agonist reduced the level of sphinganine.

Table 1. Effect of pioglitazone and high-fat diet on the weight gain and plasma free fatty acid (FFA) concentration in the experimental groups of rats.

<table>
<thead>
<tr>
<th>Pioglitazone</th>
<th>Standard diet</th>
<th>High-fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Weight gain</td>
<td>70.4±23.4</td>
<td>86.2±12.3</td>
</tr>
<tr>
<td>Plasma FFA concentration</td>
<td>198±51</td>
<td>110±16 *</td>
</tr>
</tbody>
</table>

Values are grams and nmol/ml ± SD for weight gain and plasma FFA concentration respectively (n=10).
* p<0.05 vs. the control group fed standard diet, # p<0.05 vs. the control group fed high-fat diet.
Sphinganine in the soleus and WG. High-fat diet decreased the content of sphingosine in WG and did not affect its level in the soleus and RG. In rats fed on the standard diet administration of pioglitazone increased the content of sphingosine in the soleus and had the opposite effect in the WG. In the high-fat fed group treatment with the PPARγ agonist slightly reduced the level of sphingosine in the soleus, but not in other examined muscles. High-fat diet increased the content of S1P in all muscles, with the effect being most evident in the soleus. Administration of pioglitazone to rats maintained on the standard chow markedly reduced S1P level in all examined muscle types. However, in high-fat fed animals
Fig. 2. Effect of pioglitazone and high-fat diet on the content of sphinganine, sphingosine and sphingosine-1-phosphate (S1P) in skeletal muscles. Rats were fed either the standard chow (white bars) or the high-fat diet (grey bars). Values are means ± SD, n=10. RG – red section of the gastrocnemius, WG – white section of the gastrocnemius, * p<0.05 vs. the control group fed standard diet, # p<0.05 vs. the control group fed high-fat diet, & p<0.05 vs. the respective value in the soleus, $ p<0.05 vs. the respective value in the RG.
the PPARγ agonist increased the content of S1P in the RG. The level of S1P in the soleus and WG was not significantly altered by pioglitazone.

The activity of sphingomyelinases (Fig. 3)

In the control animals maintained on the standard chow the activity of N-SMase was significantly lower in the RG comparing to the soleus and WG. High-fat diet markedly reduced the enzyme activity in all examined muscles. Administration of pioglitazone to rats fed on the standard chow increased the activity of N-SMase in the soleus and RG. However, in the case of high-fat fed

![Image](image.png)

**Fig. 3.** Effect of pioglitazone and high-fat diet on the activity of neutral (N-SMase) and acid (A-SMase) sphingomyelinase in skeletal muscles. Rats were fed either the standard chow (white bars) or the high-fat diet (grey bars). Values are means ± SD, n=6. RG – red section of the gastrocnemius, WG – white section of the gastrocnemius, * p<0.05 vs. the control group fed standard diet, & p<0.05 vs. the respective value in the soleus, $ p<0.05 vs. the respective value in the RG.
animals the PPARγ agonist did not alter the enzyme activity in either muscle. In the control animals fed on the standard chow the activity of A-SMase was significantly lower in the WG comparing to the soleus and RG. High-fat diet reduced the activity of the enzyme in all examined muscles. Pioglitazone did not affect A-SMase activity in either muscle irrespectively of the diet.

The activity of ceramidases (Fig. 4)

The activity of Al- and N-CDase was similar in all examined muscles. High-fat diet increased the activity of N-CDase in the soleus and did not affect it in

![Figure 4](image-url)

Fig. 4. Effect of pioglitazone and high-fat diet on the activity of alkaline (Al-CDase) and neutral (N-CDase) ceramidase in skeletal muscles. Rats were fed either the standard chow (white bars) or the high-fat diet (grey bars). Values are means ± SD, n=6. RG – red section of the gastrocnemius, WG – white section of the gastrocnemius, * p<0.05 vs. the control group fed standard diet, # p<0.05 vs. the control group fed high-fat diet.
other examined muscles. Administration of pioglitazone to rats fed on the standard chow elevated the enzyme activity in the RG. In high-fat fed group the drug reduced the activity of N-CDase in the soleus and induced the opposite effect in the WG. The activity of AI-CDase in the soleus and WG was not affected by neither high-fat diet nor pioglitazone. In the RG the enzyme activity was reduced by high-fat diet and increased by administration of pioglitazone.

**DISCUSSION**

The high-fat diet increased the content of sphingomyelin in all examined muscles. This effect was likely a result of reduced rate of SM degradation to ceramide, since the activity of both SMase isoforms was markedly inhibited by the high-fat diet. Increased dietary fat intake also induced the accumulation of ceramide in the soleus and RG. This is a surprising observation considering the abovementioned data on the activity of SMases. In the RG the reduced ceramide formation from sphingomyelin could be counterbalanced by the observed decrease in the activity of AI-CDase. This was not the case in the soleus where the activity of N-CDase was elevated by high-fat diet. However, the changes in the activity of ceramidases did not affect the content of sphingosine which indicates that the rate of ceramide deacylation was not altered significantly. In view of the above data, high-fat diet induced accumulation of muscle ceramide could not be explained by changes in the activity of SMases and/or CDases. Therefore, the most likely explanation of this phenomenon is the enhanced rate of ceramide synthesis *de novo* caused by increased lipid availability. This is supported by the increased content of sphinganine (an intermediate in *de novo* synthesis of CER) in the soleus and elevated concentration of plasma FFA in high-fat fed animals.

There are very few data in the literature regarding the effects of increased dietary fat intake on the activity of the examined enzymes and the content of ceramide in different tissues. Yang *at al.* (28) found a marked reduction in the activity of N- and A-SMase as well as N-CDase in the colonic mucosa of rats fed on a high-fat diet. On the other hand, Geelen and Beynen (29) reported an increase in the activity of N- and A-SMase in the liver of high-fat fed rats, which indicates that this response depends on the tissue type. Unfortunately there are no similar studies relating to muscles. Todd *et al.* (30) found that the content of ceramide in rat skeletal muscle is increased by high-fat diet, which is in line with the results of our study. Similar effect was reported in human and rat skeletal muscles after intravenous injection of lipid emulsions (31, 32). However, Lee *et al.* (33) did not observe changes in the level of muscle CER in rats subjected to a high-fat diet.

Our study demonstrated that pioglitazone reduced the content of ceramide in the soleus and RG irrespectively of dietary fat intake. This effect could not be
attributed to the decreased rate of CER formation from sphingomyelin, since the activity of A-SMase was not affected by pioglitazone, irrespectively of the diet. Moreover, in rats fed on the standard chow the administration of PPARγ agonist even increased the activity of N-SMase in the soleus and RG. In the latter muscle pioglitazone-induced decrease in the content of ceramide, at least in part, could be a result of increased rate of its deacylation. This is supported by the fact that PPARγ agonist activated N- and Al-CDase in the RG of rats fed on the standard and high-fat diet respectively. However, this activation did not translate into the increase in sphingosine content which indicates that the rate of ceramide degradation was not significantly altered. Nevertheless, in the soleus the activity of CDases was either unaltered or even decreased after pioglitazone treatment. In view of the above data, it is not likely that the observed reduction in the content of muscle ceramide was a result of its decreased formation from sphingomyelin or increased rate of deacylation to sphingosine. In our study administration of pioglitazone reduced the concentration of plasma FFA, which is a well-documented effect of thiazolidinediones (34, 35). Moreover, Ye et al. (36) found that rosiglitazone reduces fatty acid uptake in rat skeletal muscles. Therefore, considering the above data on the activity of SMases and CDases, the most likely explanation for the pioglitazone-induced decrease in the muscle CER content is the reduction in the rate of ceramide synthesis \textit{de novo} due to limited availability of plasma FFA.

There is very few data in the literature concerning the effect of thiazolidinedione administration on CER level in muscle tissue. Our results are in line with reports showing that treatment with troglitazone reduces ceramide content in mice soleus (19) and in the heart of Zucker diabetic fatty rats (37). Similarly to us, the authors of the above studies concluded that this effect was a result of the reduction in the rate of ceramide synthesis \textit{de novo}. However, it should be noted that Lessard et al. (18) showed that rosiglitazone increased the content of CER in the soleus of obese Zucker rats.

A number of studies showed that ceramide inhibits insulin-stimulated glucose uptake and GLUT4 translocation. The mechanism of this action involves inhibition of several intermediates in the insulin signaling pathway, namely insulin receptor substrate 1, phosphatidylinositol 3-kinase and Akt/protein kinase B (4). As already mentioned in the introduction, ceramide level was found to be increased in skeletal muscles of diabetic and insulin resistant humans and rodents. The results of our study indicate that decrease in the content of ceramide may be one of the mechanisms by which pioglitazone improves insulin sensitivity in skeletal muscles. The observed reduction in CER content was moderate. However, it was shown that relatively small changes in the level of this sphingolipid may significantly affect insulin signaling (4).

Administration of pioglitazone also affected the content of S1P in skeletal muscles. However, the agonist induced different effects in animals fed on the standard chow (marked reduction in S1P level) and on the high-fat diet (no
change or increase in S1P level). Nevertheless, we can only speculate on the mechanism of this phenomenon. A plausible explanation is that pioglitazone affected the activity of one or more of the enzymes involved in S1P metabolism, namely sphingosine kinase, sphingosine-1-phosphate lyase and sphingosine-1-phosphate phosphatase (3). Unfortunately, there is no other data in the literature regarding the effect of thiazolidinediones on the content of S1P or the activity of enzymes of its metabolism.

An interesting finding of our study is that the influence of pioglitazone on sphingolipid metabolism largely depended on the muscle type. Administration of PPARγ agonist induced much stronger effects in the oxidative muscles (soleus and RG) than in the glycolytic one (WG). Differences in the response to thiazolidinediones between various muscle types were found also by other investigators (38, 39). A plausible explanation for this phenomenon is the difference in PPARγ expression, which actually was reported by some investigators (40).

In summary, we found that pioglitazone reduced the content of ceramide in the oxidative muscles (soleus and red section of the gastrocnemius), but did not affect it in the glycolytic muscle (white section of the gastrocnemius) independently of dietary fat intake. This effect was likely a result of reduced rate of de novo ceramide synthesis in the oxidative muscles due to decreased availability of plasma free fatty acids. The results of our study indicate that reduction in ceramide level may be one of the mechanisms by which pioglitazone improves skeletal muscle insulin sensitivity.

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REFERENCES


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