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The effect of enterolactone on sphingolipid pathway and hepatic insulin resistance development in HepG2 cells

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ABSTRACT

Aims: Obesity and type 2 diabetes mellitus, correlate with increased tissue concentration of sphingolipids, which directly interfere with insulin signaling pathway. Phytoestrogens are a group of plant-derived compounds that have been studied in the case of metabolic disorders treatment. Therefore, the aim of this study was to ascertain whether enterolactone (ENL), a commonly known phytoestrogen, may affect sphingolipid metabolism and decrease hepatic insulin resistance development in a lipid overload state.

Main methods: The study was conducted on HepG2 cells incubated with ENL and/or palmitic acid (PA) for 16 h. Intra- and extracellular sphingolipid concentrations were assessed by high performance liquid chromatography. The expression of sphingolipid pathway enzymes, apoptosis and insulin signaling pathway proteins and glucose metabolism regulators were evaluated by Western Blot.

Key findings: In HepG2 cells, a considerable augmentation of intracellular ceramide and sphingosine concentration in ENL with PA group were indicated with simultaneous increase in extracellular ceramide concentration. The ENL treatment increased expression of selected enzymes from de novo ceramide synthesis pathway with lower expression of ceramide transfer protein. We also observed a decreased expression of insulin-stimulated phosphorylation of AKT and AMPK after exposure to ENL with PA. Our research demonstrated that ENL with PA resulted in an increased expression of caspase-3.

Significance: Enterolactone, in a higher fatty acids availability, led to the development of hepatic IR in HepG2 cells. This phenomenon may be the result of elevated intracellular ceramide accumulation caused by increased de novo synthesis pathway what led to enhanced apoptosis of HepG2 cells.

1. Introduction

Global epidemics of obesity and type 2 diabetes mellitus (T2DM) spread extensively during the past two decades, particularly among Western societies, mainly due to poor eating behaviors and diet quality [1,2]. It is commonly known that one of the most fundamental factors associated with development of those two metabolic disorders is insulin resistance (IR) [3]. Considering the clinical definition, IR is a condition with an impaired insulin action and response in liver, adipose tissue and skeletal muscles. Obesity-related hepatic insulin resistance contributes to higher plasma concentrations of both insulin and glucose. Hyperinsulinemia and hyperglycemia result in intensified intrahepatic lipogenesis and, therefore, increased intracellular lipid content in hepatocytes [4]. An excessive concentration of lipid compounds impairs oxidation pathway and leads to intensified fatty acids esterification into various lipid fractions e.g. sphingolipids, which constitute a class of lipids involved in cell signaling pathways or cell-to-cell recognition and interactions [5,6]. Although numerous components represent this class of lipids, ceramides (CER), sphingosine (SFO) and sphinganine (SFA) are considered to be the most significant. So far, numerous studies have demonstrated that occurrence of metabolic diseases e.g. type 2 diabetes mellitus are widely associated with an increased tissue concentration of

Abbreviations: T2DM, type 2 diabetes mellitus; IR, insulin resistance; ENL, enterolactone; PA, palmitic acid; AKT, pAKT, protein kinase B, phosphorylated Akt; GSK, pGSK, glycogen synthase kinase, phosphorylated GSK; AMPK, pAMPK, 5′AMP-activated protein kinase, phosphorylated AMPK; CER, ceramides; SFO, sphingosine; SFA, sphinganine; SDG, secoisolariciresinol diglucoside; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GLUT-2, insulin-regulated glucose transporter type 2; SPTLC1, 2, serine palmitoyltransferase 1, 2; LASS4, 6, ceramide synthase 4, 6; PHCA, ceramidase; CERT, ceramide transport protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; WB, Western Blot

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sphingolipids, as biomarkers of diabetes [7–9]. Although the majority of studies concerning sphingolipids and insulin resistance are based on skeletal muscles, several lines of evidence have indicated that excessive accumulation of sphingolipids is also correlated with the development of IR in hepatocytes [10,11]. It is noteworthy that modifications in sphingolipid metabolism correlated with insulin signaling pathway may be very promising for future treatment of insulin-related disorders. Phytoestrogens are a group of plant-derived compounds represented by several major classes e.g. lignans such as enterodiol or enterolactone (ENL) [12]. Despite the fact that some phytoestrogens have been well-identified and widely studied with respect to insulin resistance or T2DM reduction, research considering lignans and these two metabolic disorders were not conducted on liver. Zhou et al. have proven that ENL, the major biologically active metabolite of dietary mammalian lignans, has a prominent anti-diabetic effect in skeletal muscles, which was shown both in vitro on L6 cell line and in vivo in db/db mice [13]. Furthermore, in vivo experiments carried out on male C57BL/6J mice indicated that some flaxseed lignans e.g. secoisolariciresinol diglucoside (SDG) may alleviate the development of hepatic insulin resistance [14]. Additionally, there are no studies presenting a correlation between an immense group of lignans and sphingolipid metabolism. Thus, the current study was undertaken in order to assess, whether enterolactone affects metabolism of sphingolipids and, therefore, contributes to the development of insulin resistance in HepG2 cells, a human liver immortalized cell line.

2. Materials and methods

2.1. Cell culture

Human liver hepatocellular carcinoma cells (HepG2/C3A) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Polgen) and 1% antibiotic/antimycotic (penicillin/streptomycin). The cells were cultured for 5 days at 37 °C in a humidified atmosphere of 5% CO_2 in air upon reaching 70% confluency. During this period the medium was replaced every 48 h. After 5 days the cells were seeded in 6 well plates and cultured in standard growth medium to achieve 90% confluence.

2.2. Experimental procedure

The experiment was conducted on three-hour serum-starved cells. Palmitic acid was dissolved in a solution of ethanol and 1 M NaOH, heated to 70 °C, mixed with fatty-acid-free bovine serum albumin (2% BSA) and diluted in DMEM as it was described previously [10]. The HepG2 cells were divided into four experimental groups, which were incubated for 16 h at 37 °C in 5% CO_2 atmosphere. The first one was a control group incubated only with DMEM supplemented with BSA. In the second group the cells were incubated in the same medium as the control group and in presence of Enterolactone (ENL, 50 μM). The third group was incubated with palmitic acid (PA, 0.5 mM) and the fourth group was treated with ENL combined with PA. Moreover, in the second set of experiments, the same four experimental groups were incubated with the addition of 100 nM insulin (NOVORAPID). At the end of the procedures the cells were washed three times with ice-cold PBS and homogenized in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors (Roche Diagnostics GmbH). Furthermore, after incubation period a small amount of postincubation medium were collected and frozen in the temperature of liquid nitrogen. In all the samples total protein concentration was estimated with bicinchonic acid (BCA) assay.

2.3. Immunoblotting analyses

The Western Blot technique was used to determine the expression of selected proteins and their phosphorylated forms directly involved in insulin signaling pathway (AKT, pAKT, GSK, pGSK), the expression of sphingolipid pathway enzymes (SPTLC1, SPTLC2, LASS6, LASS4, PHCA), as well as apoptosis pathway protein (caspase-3) and glucose metabolism regulator (AMPK, pAMPK). Moreover, the expression of glucose transporter 2 (GLUT-2) and ceramide transport protein (CERT) were also determined using this technique as previously described in details by Konstantynowicz-Nowicka et al. [10]. Briefly, the cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and after blocking with 5% nonfat dry milk, incubated with primary antibodies of intents at 4 °C overnight. The blots were subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibodies and received signals were assessed densitometrically using the visualization EQ ChemiDoc (Bio Rad) system. The expression of all the proteins was standardized to the GAPDH (Santa Cruz Biotechnology, USA) expression and the control was set as 100%.

2.4. Intracellular and extracellular sphingolipid analyses

In brief, the contents of ceramide and its metabolites namely sphingosine and sphinganine were determined using a high performance liquid chromatography (HPLC) according to the method developed by Min et al. [15], as previously described by Konstantynowicz-Nowicka et al. [10]. The lipids from both HepG2 cells and samples of the medium, were extracted in the presence of internal standard namely C17-sphingosine. Next, an aliquot of the lipid extract was transported to a fresh tube containing internal standard (N-palmitayl-D-erythro-spingosine) and subjected to alkaline hydrolysis to deacylate ceramide to sphingosine. Two sphingolipid metabolites released from ceramide during analysis process, the free sphinganine and sphingosine, were next converted to their o-phthalaldehyde derivatives and analysed using standard HPLC system equipped with a fluorescence detector and C-18 reversed-phase column. The concentration of estimated sphingolipids was expressed in nanomoles per protein concentration in particular sample.

2.5. Data analysis

The data are expressed as mean values ± SD based on six independent determinations. Statistical difference between groups was tested two-way analysis of variance (ANOVA), using Statistica 10 (StatSoft, Krakow, Poland). The results were considered to be statistically significant at $P \leq 0.05$.

3. Results

3.1. Effects of HepG2 incubation with palmitic acid and/or enterolactone on the intracellular sphingolipid concentration and ceramide transport protein expression

Substantial changes were observed in HepG2 cells sphingolipids content in response to PA and/or ENL treatment during 16 h incubation. There was a considerable increase in the ceramide (CER) concentration in the PA incubated group (PA: +32%, $P < 0.05$, Fig. 1C) and PA combined with ENL (PA + ENL: 43.02%, $P < 0.05$, Fig. 1C) compared with the control group. Furthermore, we observed a significant increase in the CER concentration in the PA incubated group as well as PA combined with ENL compared with the ENL alone incubated group (PA: +11.92%, $P < 0.05$; PA + ENL: +24.76%, $P < 0.05$, Fig. 1C). We noticed a significant increase in the intracellular sphinganine (SFA) concentration in all the examined groups (ENL: +45.59%, $P < 0.05$; PA: 19.44%, $P < 0.05$; PA + ENL: +21.89%, $P < 0.05$, Fig. 1B) compared with the control group. Moreover, sphingosine (SFO) content in HepG2 cells increased significantly in the PA combined with ENL (PA + ENL: 20.44%, $P < 0.05$, Fig. 1A) group. We also observed a

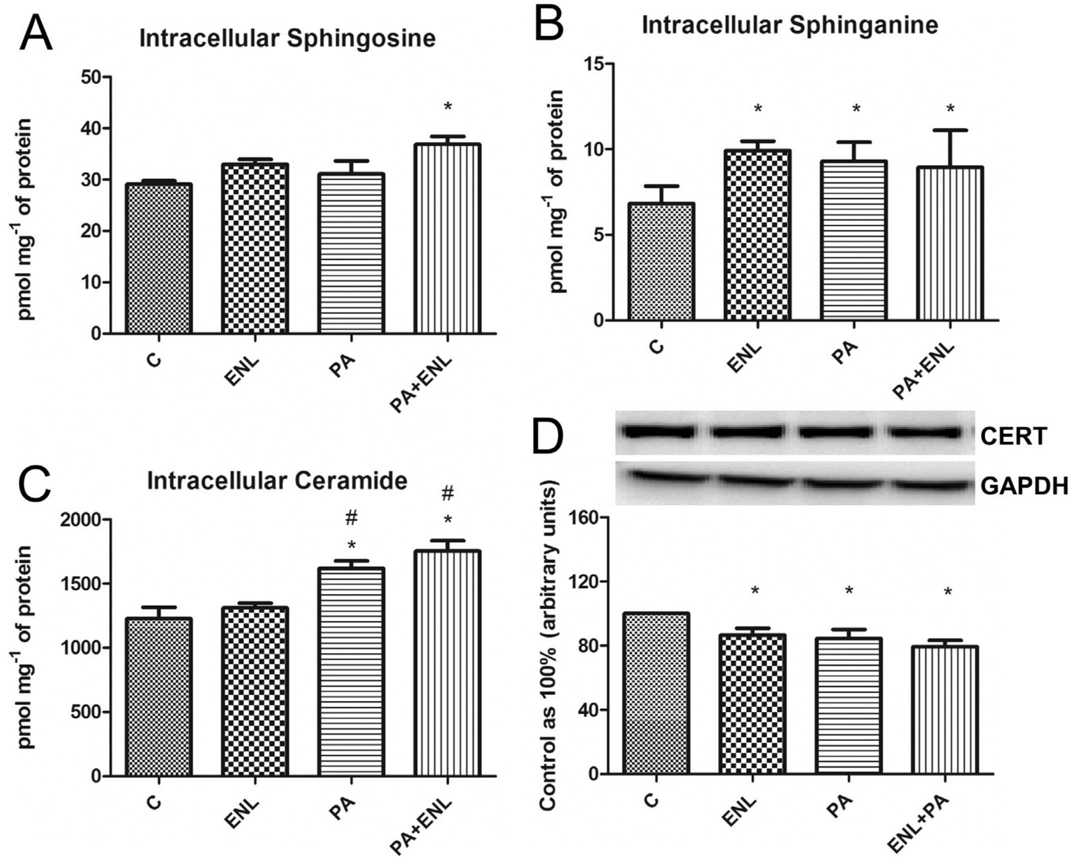

Fig. 1. Intracellular contents of sphingosine (A), sphinganine (B), ceramide (C) and expression of intracellular ceramide transport protein (D) in HepG2 cells. The cells were incubated in the absence or presence of PA (0.5 mM) and/or ENL (50 μM), as it was described in details in the Materials and methods section. All the measurements were performed after 16 h incubation. Sphingolipid content in HepG2 cells was measured by HPLC method whereas the protein expression in HepG2 cells was estimated using Western blot procedure. The data are expressed as the mean ± SD and are based on six independent determinations (n = 6). *$P < 0.05$ significant difference vs. control group; #$P < 0.05$ significant difference vs. enterolactone group; C — control, ENL — enterolactone, PA — palmitate, ENL + PA — enterolactone + palmitate.

significant decrease in the expression of CERT in HepG2 cells in all the examined groups compared with the control group (ENL: −13.7%, $P < 0.05$; PA: −16.44%, $P < 0.05$; PA + ENL: −15.84%, $P < 0.05$, Fig. 1D).

3.2. *Effects of HepG2 incubation with palmitic acid and/or enterolactone on sphingolipid metabolism enzymes*

Incubation of HepG2 cells with PA and/or ENL caused substantial changes in the expression of key enzymes involved in sphingolipid metabolism. Incubation of HepG2 cells with PA alone as well as PA combined with ENL (ENL: +5.81%, $P > 0.05$; PA: +15.37%, $P < 0.05$; PA + ENL: +26.19%, $P < 0.05$, Fig. 2C) revealed a substantial increase in the expression of serine palmitoyltransferase 1 (SPTLC1). Furthermore, in all the examined groups a significant increase in the expression of ceramide synthase 6 (LASS6) enzyme was observed (ENL: +26.46%, $P < 0.05$; PA: +22.6% $P < 0.05$; PA + ENL: +28.38%, $P < 0.05$, Fig. 2A). Moreover, the expression of ceramidase (PHCA) was significantly increased in the PA combined with ENL group (PA + ENL: +16.86%, $P < 0.05$, Fig. 2E) compared with the control group. However, the expression of serine palmitoyltransferase 2 (SPTLC2) (ENL: −6.99%, $P > 0.05$; PA: −6.67%, $P > 0.05$; ENL + PA: −4.24%, $P > 0.05$, Fig. 2D) and ceramide synthase 4 (LASS4) (ENL: −2.60%, $P > 0.05$; PA: −4.62%, $P > 0.05$; PA + ENL: −12.81%, $P > 0.05$, Fig. 2B) remained unchanged in all the examined groups.

3.3. *Effects of HepG2 incubation with palmitic acid and/or enterolactone on the expression of insulin signaling pathway proteins*

There was a significant decrease in the expression of insulin-stimulated phosphorylation of AKT (PA + INS: −20.51%, $P < 0.05$; PA + ENL + INS: −20.70%, $P < 0.05$, Fig. 3B) in HepG2 cells. On the other hand, total expression of AKT (ENL + INS: −1.28%, $P > 0.05$; PA + INS: +2.73%, $P > 0.05$; PA + ENL + INS: −3.59%, $P > 0.05$, Fig. 3A), GSK (ENL + INS: −0.48%, $P > 0.05$; PA + INS: +2.82%, $P > 0.05$; PA + ENL + INS: +8.14%, $P > 0.05$, Fig. 3C) phosphorylated GSK (ENL + INS: +0.27%, $P > 0.05$; PA + INS: −1.87%, $P > 0.05$; PA + ENL + INS: −2.01%, $P > 0.05$, Fig. 3D) and AMPK (ENL + INS: +2.53%, $P > 0.05$; PA + INS: +5.42%, $P > 0.05$; ENL + PA + INS: +9.47%, $P > 0.05$, Fig. 3E) remained unchanged in all the examined groups. There was a significant decrease in the expression of pAMPK in HepG2 cells in groups treated with palmitate and insulin as well as PA combined with ENL and INS treated groups compared with the corresponding control group (ENL + INS: −4.99%, $P > 0.05$; PA + INS: −26.47%, $P < 0.05$; PA + ENL + INS: −20.06%, $P < 0.05$, Fig. 3F). Moreover the expression of GLUT-2 was only decreased in palmitate combined with ENL and INS group (ENL + INS: −0.09%, $P > 0.05$; PA + INS: −0.017%, $P > 0.05$; PA + ENL + INS: −11.43%, $P < 0.05$, Fig. 3G) compared with the control group.

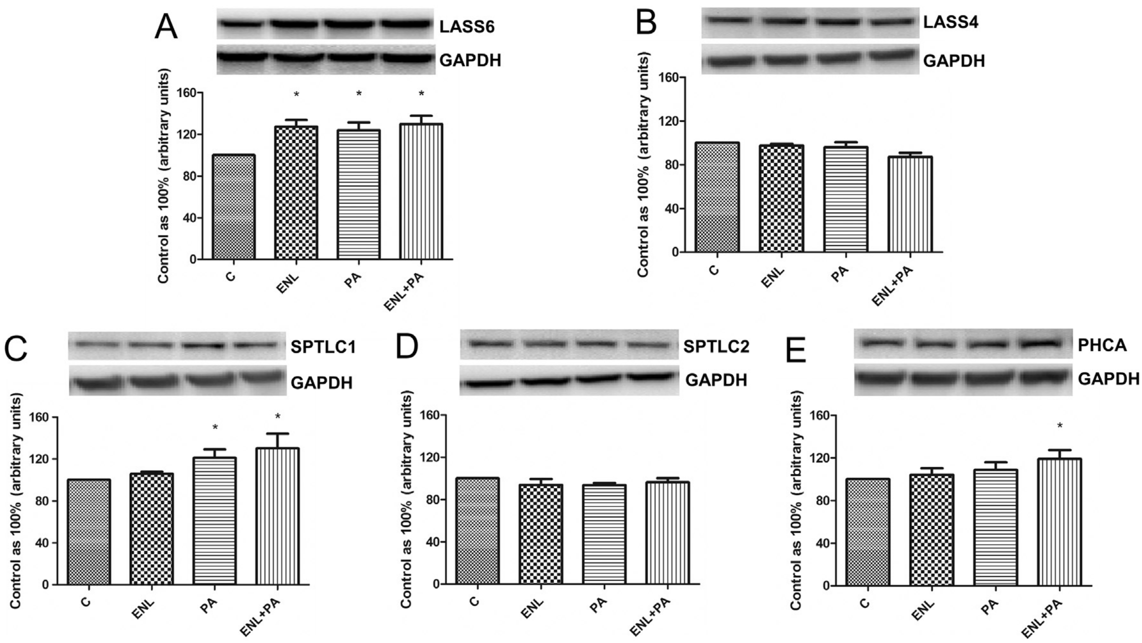

Fig. 2. The expression of proteins involved in sphingolipid metabolism i.e. LASS6 (A), LASS4 (B), SPTLC1 (C), SPTLC2 (D), and PHCA (E).
The cells were incubated in the absence or presence of PA (0.5 mM) and/or ENL (50 μM), as it was described in details in the Materials and methods section. All the measurements were performed after 16 h incubation. The protein expression in HepG2 cells was measured using Western blot method. The data are expressed as the mean ± SD and are based on six independent determinations (n = 6). *$P < 0.05$ significant difference vs. control group; C — control, ENL — enterolactone, PA — palmitate, ENL + PA — enterolactone + palmitate.

3.4. Effects of HepG2 incubation with palmitic acid and/or enterolactone on the extracellular sphingolipid content

As expected, we observed a notable increase in the ceramide content in postincubation culture media in the PA combined with ENL group in comparison with the control group (PA + ENL: +257.42%, $P > 0.05$, Fig. 4A). Moreover, the ceramide accumulation decreased significantly in the PA group compared with the group incubated with ENL alone (PA: −70.52%, $P < 0.05$, Fig. 4A). However, the accumulation of sphinganine in the culture media significantly decreased in all the examined groups (ENL: −25.7%, $P < 0.05$; PA: −47.26%, $P < 0.05$; PA + ENL: −30.94%, $P < 0.05$, Fig. 4B). Furthermore, a significant decrease of the extracellular sphingosine concentration was observed in the groups incubated with PA as well as PA combined with ENL (PA: −40.51%, $P < 0.05$; PA + ENL: −56.5%, $P < 0.05$, Fig. 4C) in comparison with the control group and in PA combined with ENL group

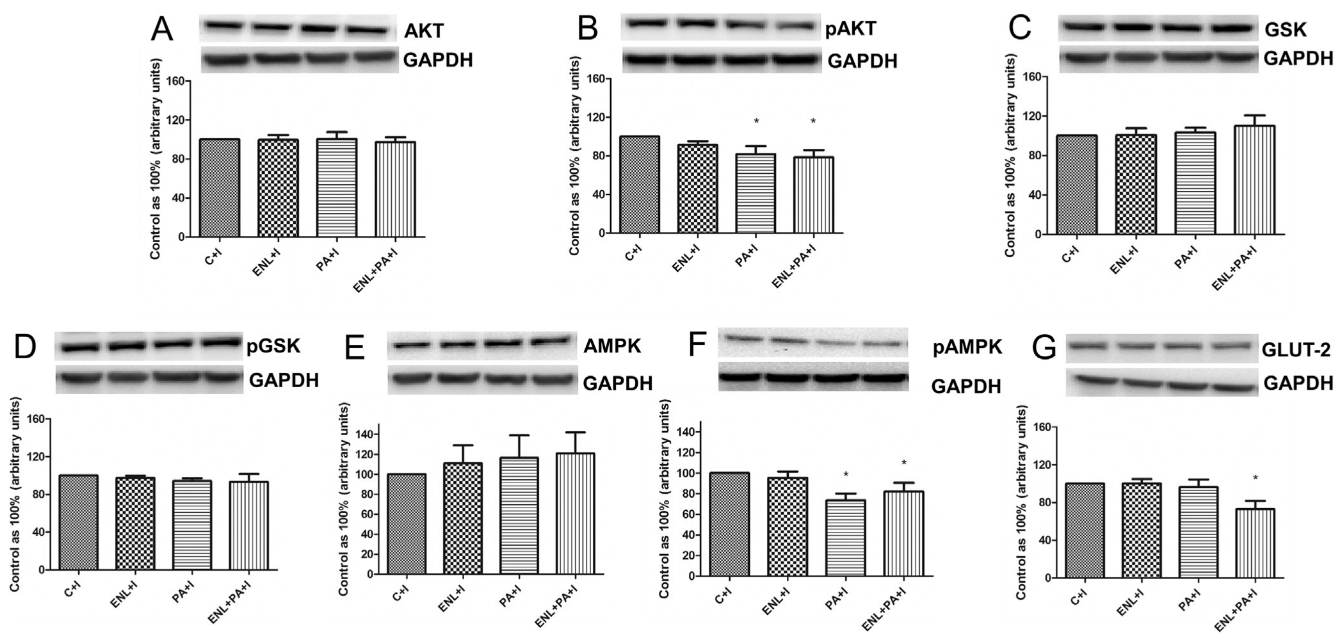

Fig. 3. The expression of proteins involved in insulin signaling pathway i.e. AKT (A), pAKT (B), GSK (C), pGSK (D), AMPK (E), pAMPK (F) and GLUT-2 (G).
The cells were incubated in the absence or presence of PA (0.5 mM) and/or ENL (50 μM), as it was described in details in the Materials and methods section. All the measurements were performed after 16 h incubation. The protein expression in HepG2 cells was measured using Western blot method. The data are expressed as the mean ± SD and are based on six independent determinations (n = 6). *$P < 0.05$ significant difference vs. control group; C + I — control + insulin, ENL + I — enterolactone + insulin, PA + I — palmitate + insulin, ENL + PA + I — enterolactone + palmitate + insulin.

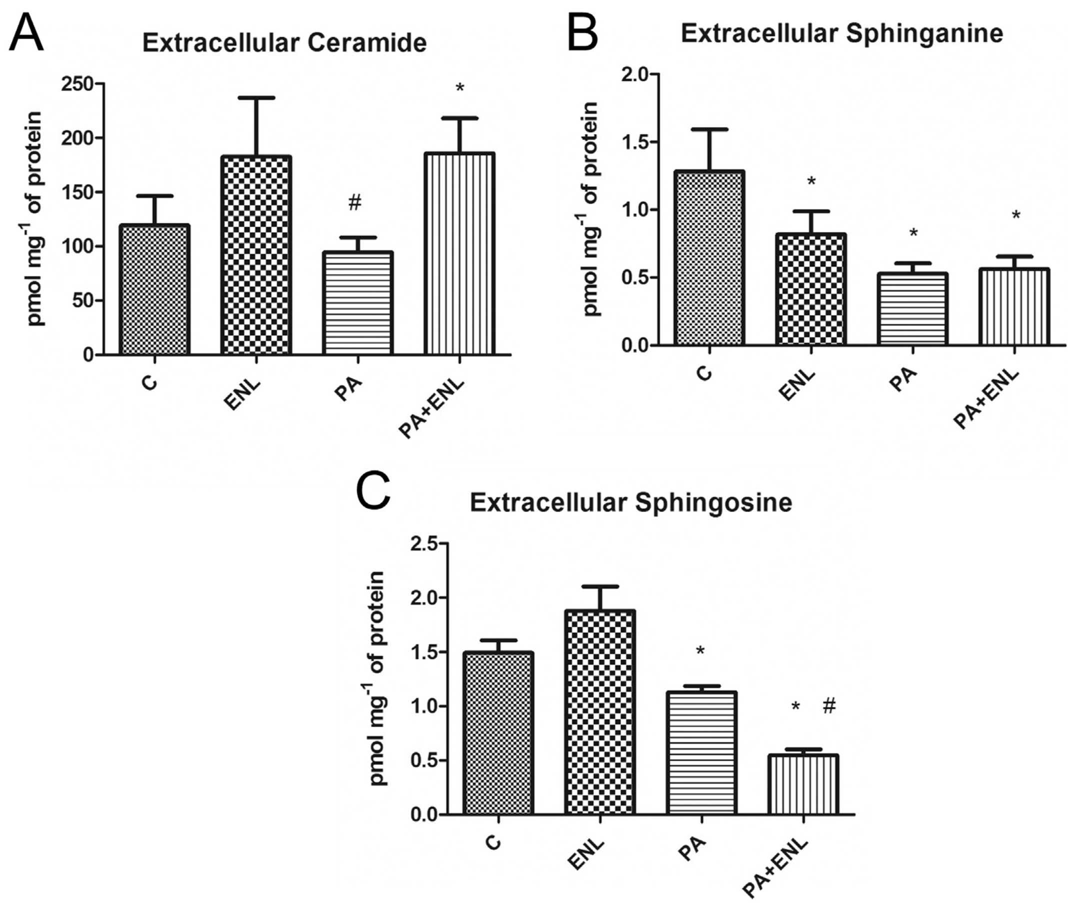

Fig. 4. Extracellular content of ceramide (A), sphinganine (B) and sphingosine (C) in postincubation media.
The cells were incubated in the absence or presence of PA (0.5 mM) and/or ENL (50 µM), as it was described in detail in the Materials and methods section. All the measurements were performed after 16 h incubation. Sphingolipid content in post incubation media was measured using HPLC. The data are expressed as the mean ± SD and are based on six independent determinations (n = 6). *$P < 0.05$ significant difference vs. control group; #$P < 0.05$ significant difference vs. enterolactone group; C — control, PA — palmitate, ENL — enterolactone, ENL + PA — enterolactone + palmitate.

compared with the ENL alone incubated group (PA + ENL: −21.33%, $P < 0.05$, Fig. 4C).

3.5. Effects of HepG2 incubation with palmitic acid and/or enterolactone on the expression of apoptotic protein (caspase-3)

The expression of protein involved in apoptosis pathway in HepG2 cells was affected during incubation with PA and/or ENL. However, only the presence of PA together with ENL caused a significant increase in the caspase-3 expression compared with the control group (PA + ENL: +21.52%, $P < 0.05$, Fig. 5A).

4. Discussion

Over the last decade, phytoestrogens have been considered as a prominent therapeutic strategy for treatment of numerous metabolic disorders including non-alcoholic fatty liver disease or type 2 diabetes mellitus [16,17]. One of the flaxseed-derived phytoestrogens, enterolactone, is deemed as a principal mammalian lignans metabolite with many promising effects e.g. anti-diabetic or anti-inflammatory [18]. Sun et al. have indicated in a study carried out on 1107 T2DM subjects that metabolites of dietary lignans, mainly ENL, led to a lower risk of type 2 diabetes mellitus occurrence [19]. The effects of enterolactone on lipid metabolism was investigated in Fortin et al. study, who demonstrated that ENL alleviates the oxidative damage of lipids after exposure to linoleic or α-linoleic acid and may diminish the accumulation of triacylglycerols (TAGs) via increasing the expression of

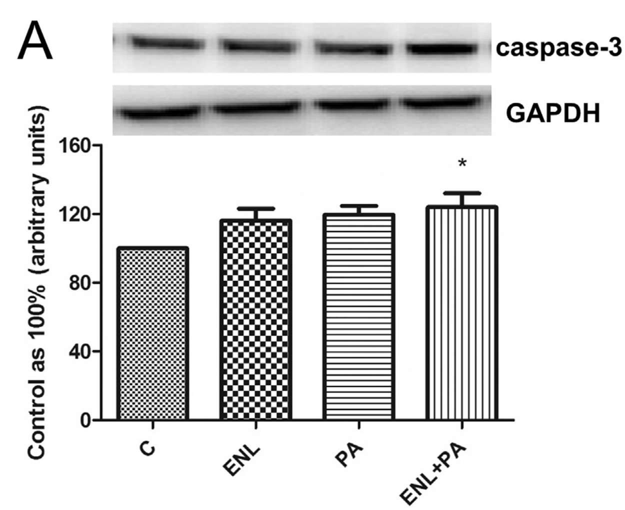

Fig. 5. Expression of protein involved in apoptosis (A).
The cells were incubated in the absence or presence of PA (0.5 mM) and/or ENL (50 µM), as it was described in details in the Materials and methods section. All the measurements were performed after 16 h incubation. The protein expression in HepG2 cells was measured using Western blot method. The data are expressed as the mean SD. and are based on six independent determinations (n = 6). *$P < 0.05$ significant difference vs. control group; C — control, ENL — enterolactone, PA — palmitate, ENL + PA — enterolactone + palmitate.

lipogenic genes in animal-derived hepatocytes [20]. Considering the fact that deposited TAGs may be esterified to the distinct fractions, we decided to assess the influence of ENL on a class of lipids that is more biologically active in comparison with other lipid fractions, namely sphingolipids [21]. Thus, in our research, conducted on HepG2 cell line, direct effect of enterolactone on sphingolipid concentration, their metabolism e.g. sphingolipid biosynthesis pathway, enzymes, and transporters was evaluated. Moreover, we hypothesized that ENL via interfering with sphingolipids metabolism may lead to the development of hepatic insulin resistance in HepG2 cell line. So far, there have been no studies concerning the influence of lignans, including enterolactone, on sphingolipid concentration and metabolism. Ceramide is widely known to be a precursor for many other sphingolipids e.g. sphingosine or sphingomyelin (SM). Hence it may be considered to be a major element of the sphingolipid metabolism pathway. Two fundamental ways of CER synthesis may be distinguished – de novo ceramide synthesis route, possible due to a condensation of two components – serine and palmitoyl-CoA, and sphingosine breakdown, so-called salvage pathway [22]. A considerable augmentation of intracellular CER concentration in enterolactone together with palmitic acid (PA) group was indicated in HepG2 cells. Additionally, our study demonstrated a notable increase in the expression of enzymes involved in de novo synthesis, including two major isoforms in HepG2 cells — SPTLC1 and LASS6 in ENL combined with palmitic acid group. Furthermore, ENL combined with PA showed increased concentration of sphinganine, being a precursor in CER formation. Therefore, it may be assumed that ENL under conditions of elevated fatty acids availability (PA treated group) led to an intensified de novo ceramide synthesis pathway. Considering the intracellular content of another examined sphingolipid, higher concentrations after exposure to both ENL and PA were noticeable in sphingosine fraction, which is a metabolite from ceramide breakdown through salvage pathway [23]. Therefore, we may extrapolate that an elevated concentration of SFA and SFO, led to an intensified biosynthesis of ceramide on de novo route and subsequently to an augmented concentration of ceramide in human liver immortalized cells. Interestingly, our findings demonstrated, in all the examined groups, a lower expression of ceramide transfer protein (CERT), which is widely involved in endoplasmic reticulum-to-Golgi complex ceramide transferring [24]. Thus, a reduced CERT expression may lead to a less efficient ceramide transport inside the cell. Sphingolipids, such as ceramides, are known to extensively interfere with the insulin-signaling pathway through inhibition of insulin-stimulated protein kinase B (AKT/PKB) phosphorylation, which constitutes an intermediary process for the anabolic metabolism. As a result, a suppression of AKT/PKB phosphorylation leads to the impairment in insulin sensitivity [25]. In contrast, some studies have shown that inhibition of enzymes involved in sphingolipids biosynthesis effects in enhanced insulin sensitivity and improved glucose tolerance in rodent model of obesity [26]. Furthermore, Chaurasia et al. suggested that ceramide, among other sphingolipids, may be regarded as a major factor that contributes to the occurrence of insulin resistance and liver-related diseases [27]. Considering the changes in the expression of insulin signaling pathway proteins, we observed in ENL combined with palmitic acid group a lower expression of insulin-stimulated phosphorylation of AKT. Moreover, a reduced expression of pAMPK after exposure to enterolactone with PA was noticeable in HepG2 cells. Interestingly, these results may suggest that ENL through suppression of insulin signaling proteins phosphorylation led to reduction of insulin sensitivity and thus to the development of hepatic insulin resistance. Additionally, the expression of GLUT-2 (insulin-regulated glucose transporter type 2) was significantly lower in ENL combined with palmitate group, therefore it may be assumed that glucose uptake from incubation medium to hepatocytes was reduced in the condition of hepatic IR development. However, our data are in contrast with the results of Morisset et al., who have proven that lignan-rich diet, including enterolactone, may lead to an improvement in insulin sensitivity in post-menopausal women [28]. It may be speculated that the most probable explanation for this discrepancy is the use of different models, namely cell culture of malignant hepatocytes contrary to human studies conducted by Morisset et al. Our studies are in accordance with the results of Powell et al., whose in vitro study indicated that an increased intracellular ceramide synthesis correlated with alleviated insulin sensitivity in L6 muscle cells [29]. Moreover, in vivo studies conducted on Rhesus macaques have demonstrated that sphingolipids, mainly ceramide, interfere with the insulin-signaling pathway. This contributes to the reduction of insulin sensitivity and, therefore, may constitute an additional biomarker of systemic insulin resistance development [30]. Considering our findings, it may be ascertained that ENL enhanced the hepatic insulin resistance via the mechanism of increased sphingolipid concentration in the palmitate-rich condition in HepG2 cells. It is worth noting that not only does an intracellular accumulation of CER lead to the development of IR but it also may cause other conditions e.g. cell apoptosis. Ordonez et al. indicated that an augmented content of ceramide significantly correlated with an alleviated initiation of apoptosis in HepG2 cell line [31]. Our research demonstrated that the action of enterolactone together with palmitic acid resulted in an increased expression of caspase-3, a crucial apoptosis marker. Considering this phenomenon, it may be speculated that in cancer cells accumulation of ceramide and further development of apoptosis after exposure to enterolactone and palmitate constitutes a route for these pathological cells elimination. Our findings are in agreement with Hausott et al., who have indicated that in vitro usage of lignans lead to the cell apoptosis in SW480 colon carcinoma cells [32]. Although the cell is regularly exposed to an excessive accumulation of harmful components, it presents numerous protective mechanisms e.g. outward efflux [33]. In our research it was revealed that in human liver immortalized cell line an exposure to enterolactone and palmitic acid led to a higher extracellular concentration of CER in comparison with the control group. Therefore, it may be ascertained that HepG2 cells are defending themselves against excessive ceramide accumulation through an augmented efflux of CER to the incubation medium, thus may prevent the hepatic insulin resistance occurrence. Although the only compound with a significantly higher extracellular concentration was ceramide, it may be hypothesized that an augmented mechanism of apoptosis may also be the result of increased extracellular sphingolipid concentration. A significant decrease in concentration of other two sphingolipids — SFA and SFO in postincubation media was noticeable in ENL combined with palmitate group. Considering these results, it may be assumed that ceramide, as the most harmful compound, was transported outside the HepG2 cell. Although in HepG2 a compensatory defense mechanism exists, it is not as efficient as it may be regarded.

5. Conclusions

Although the enterolactone is considered to be an anti-diabetic factor, under the conditions of elevated fatty acids availability this widely-studied phytoestrogen led to the development of hepatic insulin resistance in human hepatocellular carcinoma cells. It may be hypothesized that IR occurrence after the enterolactone treatment was caused by excessive CER accumulation through intensified de novo ceramide synthesis pathway. What is more, the increased deposition of this bioactive sphingolipid metabolite, enhanced the apoptosis in HepG2 cells. These findings may provide scientific background for implementation of safer methods of natural origin acting therapeutically in the treatment of liver cancer, especially in obese patients. However, these data should be confirmed by in vivo studies to become a new therapeutic factor augmenting liver cancer cell death.

Acknowledgements

Author contributions

TC, NI and KKN were responsible for the conception and design of the study, analysis and interpretation of data, design of the article and drafting the article. KB and KD were involved in experiment conduction. AC and KKN were responsible for analysis and interpretation of data and revised the manuscript critically. All authors gave final approval.

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Conflict of interest

All authors declare that they have no competing interests.

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