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The effect of enterolactone on liver lipid precursors of inflammation

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ABSTRACT

Aims: The aim of this study was to assess the effects of enterolactone (ENL) on lipid fractions fatty acids composition affecting hepatocyte inflammation development.
Main methods: The experiments were conducted in HepG2 cells incubated with ENL and/or palmitic acid (16 h). Intracellular contents of free fatty acids (FFA), di- (DAG) and tri- (TAG) acylglycerol as well as their fatty acids compositions were assessed by Gas-Liquid Chromatography. Moreover, the ω-6/ω-3 ratios in the above mentioned lipids fractions were estimated. The expression of proteins involved in eicosanoids and prostanoids production (COX-2, 15-LOX), inflammatory process (TNFα), as well as the proteins participating in the desaturation (SCD 1) and elongation (Elovl 3, Elovl 6) of fatty acids were evaluated by Western Blot.
Key findings: Enterolactone modified fatty acids composition in FFA, DAG and TAG fractions. In conjunction with lipid overload, it increased the content of ω-6 more than ω-3 PUFA. Moreover, it enhanced the expressions of Elovl 3, Elovl 6, COX-2 and TNFα, whereas it had no influence on SCD 1 and 15-LOX level.
Significance: Our study revealed that the supplementation with ENL affected intracellular hepatic composition of saturated as well as unsaturated fatty acids in each of the investigated lipid fractions. Based on the shift in the ω-6/ω-3 balance towards ω-6, as well as the increase in COX-2 and TNFα protein expressions, we may postulate a pro-inflammatory nature of the examined polyphenol. Moreover, our findings could prove to be useful in the future research in the topic of widespread diseases such as NASH.

1. Introduction

Disturbances in the fatty acids metabolism might be the cause of many widespread disorders such as obesity, type 2 diabetes mellitus (T2DM) or non-alcoholic fatty liver disease (NAFLD). In most of the above mentioned cases, impaired lipid homeostasis is related to either inappropriate diet or metabolic dysfunctions. This leads to an excessive lipid accumulation and results in prolonged inflammatory states. The liver is not well-suited for handling an excessive fatty acid influx over prolonged period of time, hence, hepatocytes' malfunctions often develop [1]. It is still being discussed which fatty acid or lipid fraction is particularly responsible for the development of the inflammation and deterioration observed in NAFLD [2]. Nevertheless, it is known that the excessively accumulated fatty acids may constitute substrates for inflammatory pathways and be metabolized by lipoxygenase to leukotrienes or by cyclooxygenase to prostanoids [3,4]. The above are the base for the development of local inflammatory processes which in turn lead to the impaired tissue functioning and oxidative stress [5,6]. The actual content of the lipids fractions accumulated in the liver strongly depends on the composition of a diet [5,7]. Despite the undoubted role of n-6 polyunsaturated fatty acids (PUFA) in the production of arachidonic acid (AA), plenty of studies in human subjects failed to prove the relationship between the increased concentrations of these fatty acids in blood plasma and elevated levels of inflammatory markers [8,9]. Western style diet, rich in saturated fatty acids (SFA), leads to the storage of the consumed SFA (mainly palmitic acid) in different lipid fractions and results in an increased apoptosis rate, ER stress, and inflammation in the liver [10,11]. However, not only the type of lipids accumulated, but also their saturation and elongation status seem to be of vital importance, since that may indicate the direction of metabolic changes. As indicated in studies conducted on mice, an increased expression of stearyl-CoA desaturase 1 (SCD1) stimulated the production

Abbreviations: ENL, enterolactone; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TAG, triacylglycerols; DAG, diacylglycerols; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PA, palmitic acid; SREBP, sterol regulatory element-binding protein; FFA, free fatty acids; TNFα, tumor necrosis factor α; Elovl 3 and 6, elongases 3 and 6; SCD 1, stearyl-CoA desaturase 1; THP-1, human monocyte cell line; 15-LOX, lipoxygenase 15; T2DM, type 2 diabetes mellitus; COX-2, cyclooxygenase 2; ALA, α linolenic acid; LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid

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of monounsaturated fatty acids (MUFA) in a lipid overload state [11]. The above led to liver steatosis and NAFLD development [11]. Interestingly, high fat diet increased the activity of elongases and promoted the accumulation of *n*-3 and *n*-6 PUFAs in the liver [12]. Furthermore, increased activities of very long chain fatty acid elongases, as observed in patients with nonalcoholic steatohepatitis (NASH), but not NAFLD, showed the role of these enzymes in the deterioration of steatosis [13,14]. However, safe methods for NAFLD treatment via modulation of the accumulated fatty acids composition are still not known. Therefore, anti-inflammatory substances potentially affecting inflammatory pathways, lipid accumulation and/or saturation status, i.e. polyphenols, seem to be a promising subject of study [15,16]. One of the candidates might be enterolactone (ENL), since it affects lipid metabolism and has anti-oxidative properties [17,18]. Nevertheless, despite its potential, ENL is still a relatively poorly examined polyphenol. Therefore, in the present study we estimate its influence on lipids fatty acids composition, saturation and elongation processes as well as the induction of the inflammation in palmitate overloaded hepatocytes.

2. Materials and methods

2.1. Cell culture

The HepG2/C3A cells obtained from ATCC (American Type Culture Collection) were grown in a standard growth medium (DMEM-Dulbecco Modified Eagle Medium, PAN-Biotech, Germany) enriched with 10% fetal bovine serum (FBS, BioWest, France) and 1% penicillin/streptomycin (Sigma-Aldrich, USA) for 5 days at 37 °C in a humidified atmosphere containing 5% of CO_2. The number of passages for cells used in the experiment was in the range from passage five up to passage seven. Once they reached 70% confluence the cells were transferred to 6 well plates. The cells' viability was assessed in Bürker chamber using Trypan blue (Sigma-Aldrich, USA) staining.

2.2. Experimental groups

Briefly, after reaching 90% confluence HepG2 cells were incubated in DMEM containing 2% fatty acid-free bovine serum albumin (BSA, Sigma-Aldrich, USA) combined with palmitic acid (PA, Sigma-Aldrich, USA) dissolved in a solution of ethanol, and 1 M NaOH (Sigma-Aldrich, USA) as previously described by Konstantynowicz-Nowicka et al. [16]. Before the experiments the cells were serum-starved for 3 h. Afterwards, they were incubated in medium containing either 50 μM ENL (Sigma-Aldrich, USA) alone, 0.5 mM palmitic acid or with both ENL and PA for 16 h. The concentration and exposition time of PA and ENL used in our experiment were selected during preliminary studies and were based on studies conducted by Jansen et al. [19]. At the end, the cells were homogenized in ice-cold RIPA buffer containing protease inhibitors (Roche Diagnostics GmbH, Germany), ultrasonicated and frozen. Moreover, after the incubation period samples of post-incubation media were collected and frozen in a liquid nitrogen.

2.3. Western blotting

Total protein concentration was estimated with bicinchoninic acid assay and calibrated using BSA as a standard. Subsequently, the samples were boiled with a 2-mercaptoethanol buffer for 10 min at 95 °C. Cell lysate probes (10 μg of lysate each) were separated using 10% Criterion TGX Stain-Free precast Gel (Bio Rad, Poland) electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Then the membranes were blocked with TTBS buffer containing 5% nonfat dry milk for 90 min in a room temperature. Afterwards, membranes were immunoblotted with primary antibodies of interest and then with the appropriate, horseradish peroxidase labeled (HRP), anti-goat (for SCD1, Elovl 3 and 6) or anti-mouse (for COX-2, 15-LOX and GAPDH) secondary antibodies (Santa Cruz Biotechnology, USA). The protein expression was assayed densitometrically using ChemiDoc visualization system (Bio Rad, Poland). Equal protein loading was confirmed using Ponceau S (Sigma-Aldrich, USA) staining. The expression of all the proteins was standardized to the GAPDH (Santa Cruz Biotechnology, USA) expression and the control was set at 100%. The primary antibodies anti-: COX-2, 15-LOX, SCD1, Elovl 3 and Elovl 6 were purchased from Santa Cruz Biotechnology, USA.

2.4. Intra- and extracellular lipid analysis

Lipids from both pre- and post-incubation media were extracted with a chloroform-methanol solution using Folch method [20] and separated into: free fatty acids (FFA), DAGs and TAGs by thin-layer chromatography (TLC) [21]. Subsequently, individual fatty acid fractions were methylated in 14% methanol solution in boron trifluoride and quantified according to the retention times of standards by using Gas-Liquid chromatography procedure (GLC Hewlett-Packard 5890 Series II gas chromatography HP-innowax capillary column equipped with a flame ionization detector). Moreover, based on fatty acids composition we ascertained the sum of ω-3 and ω-6 polyunsaturated fatty acids in the investigated individual lipid fraction and ω-6/ω-3 ratio. Concentrations of estimated fatty acids species of lipid fractions were expressed in nanomoles per protein concentration in particular sample.

2.5. Data analysis

All data are expressed as the mean and standard deviation. The assumptions of the methods used in our analysis, that is normality of the data distribution (Shapiro-Wilk test) and homogeneity of the variance (Bartlett's test) were checked. Statistical differences were determined based on the results of one-way ANOVA followed by an appropriate post-hoc test (i.e. pairwise Student's *t*-test) using GraphPad Prism 7. $P < 0.05$ was accepted as statistically significant in all cases.

3. Results

3.1. Effects of HepG2 exposure to PA and/or ENL on the intracellular fatty acids composition in FFA fraction

We noticed an increase in palmitic acid (16:0) level but only in the ENL + PA-treated group (Fig. 1A; ENL + PA: +44.6%) and high arachidic acid (20:0) level after exposure to ENL (Fig. 1A; ENL: +46%). The contents of linoleic acid (18:2 ω-6) were significantly elevated in all of the experimental groups (Fig. 1A; ENL: +34.7%, PA: +17.7%, ENL + PA: +37.4%). Moreover, we revealed that in all the examined groups arachidonic acid (20:4 ω-6) content was increased in comparison to the control group. Notably, this elevation was most pronounced in the group exposed to enterolactone and was significantly higher than in the group treated with palmitate (Fig. 1A; ENL: +147.5%, PA: +52.8%, ENL + PA: +45.4%). Similarly, α linolenic acid (18:3 ω-3) levels were increased in all of the examined groups (Fig. 1A; ENL: +27.3%, PA: +15.9%, ENL + PA: +20.1%). The levels of oleic acid (18:1) rose markedly in the groups treated with ENL alone or in connection with PA (Fig. 1A; ENL: +19.3%, ENL + PA: 20.8%). Moreover, simultaneous treatment with palmitate and enterolactone caused elevation in ω-6/ ω-3 ratio in HepG2 cells, which was significantly higher in comparison to the control group as well as PA-treated group (Fig. 5A; ENL + PA: 2.4%).

3.2. Effects of HepG2 exposure to PA and/or ENL on the intracellular fatty acid composition in DAG fraction

As suspected, the level of palmitic acid (16:0) was increased in the PA and ENL + PA-treated groups (Fig. 1B; PA: +58.9%, ENL + PA: +11.3%). The level of oleic acid (18:1) rose markedly in all the

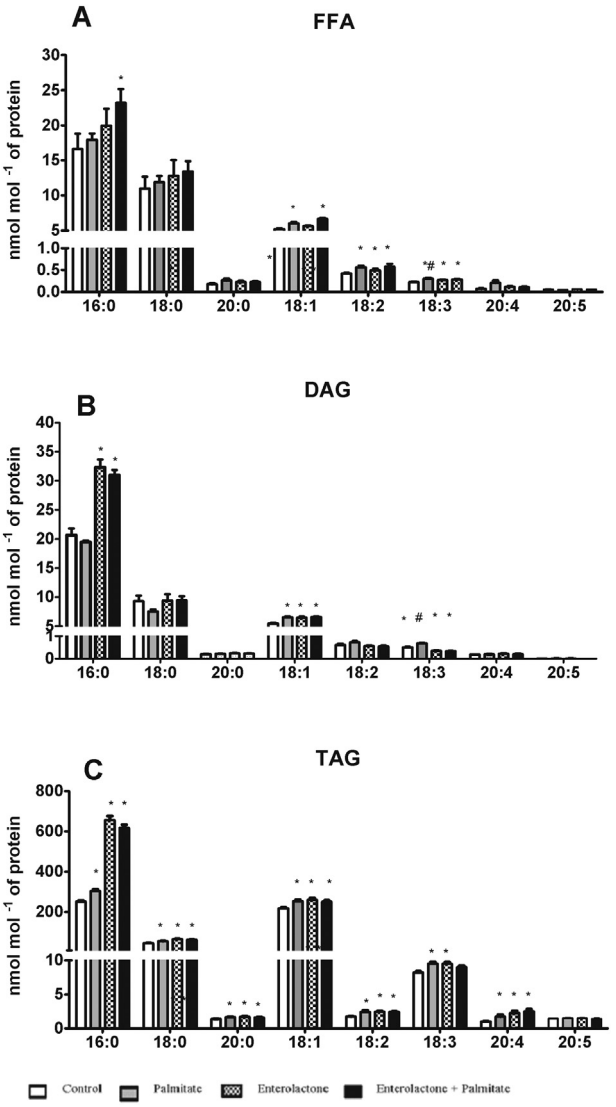

Fig. 1. The composition of the free fatty acids (A), diacylglycerols (B) and triacylglycerols (C) in HepG2 cells.
The cells were incubated with enterolactone (50 μM) alone or combined with palmitic acid (0.5 mM) for 16 h as it was described in details in Materials and methods section. Total lipid content in HepG2 cells was measured by GLC method. The data are expressed as the mean ± S.D. and are based on six independent determinations. *P < 0.05 significant difference vs control group; ** 0.05 < P < 0.01 significant difference vs control group; #P < 0.05 significant difference vs palmitate-treated group.

examined groups (Fig. 1B; PA: +19.2% ENL: 18,6% ENL + PA: 20.8%). Among all the evaluated DAG's polyunsaturated fatty acids fractions, we noticed that α linolenic acid (18:3 ω-3) level increased markedly in the group of the cells exposed to ENL alone, whereas it decreased in the PA and ENL + PA-treated groups (Fig. 1B; ENL: +35.6%, PA: −30.6%, ENL + PA: −33%). Moreover, the level of α linolenic acid (18:3 ω-3) in the ENL-group was also higher compared to the groups treated with PA. There were no significant changes in DAG's ω-6/ ω-3 ratio (Fig. 5B). The 18:1/18:0 ratio were elevated after exposure to enterolactone (Fig. 2B; ENL: 0.9; 0.29, respectively) as compared to the control group.

3.3. Effects of HepG2 exposure to PA and/or ENL on the intracellular fatty acids composition in TAG fraction

In TAG fraction ENL alone increased the levels of stearic acid (18:0) (Fig. 1C; ENL: +21%) and arachidic acid (20:0) (Fig. 1C; ENL: +20%) as compared to the control group. In addition, the exposure to PA or PA and ENL simultaneously resulted in a significant rise in SFA level. This encompassed: palmitic (16:0) (Fig. 1C; PA: +45.6%. ENL + PA: +45.6%), stearic (18:0) (Fig. 3; PA: +42.8%, ENL + PA: +38.2%), and arachidic acid (20:0) (Fig. 1C; PA: +24.0%, ENL + PA: +17.3%). Moreover, we detected a substantial increase in oleic (18:1) (Fig. 1; PA: +16.1%, ENL: +19% ENL + PA: +14.9%), linoleic (18:2 ω-6) (Fig. 1; ENL: +39.2%, PA: +41.7%, ENL + PA: +39.9%), and arachidonic acid (20:4 ω-6) content (Fig. 1; ENL: +71.9%, PA: +123%, ENL + PA: +148.9%) in all of the examined groups. The amount of α linolenic (18:3 ω-3) acid increased significantly in the groups treated with palmitate or enterolactone (Fig. 1C; PA: +15.6%, ENL: +15.6%). Furthermore, TAG's ω-6/ω-3 ratios were substantially increased in the groups treated with PA alone and PA + ENL, as compared to the control group (Fig. 5C; C: 0.2; PA: 0.3; ENL + PA: 0.3). The TAG's 18:1/18:0 ratios were considerably increased in all the examined groups (Fig. 2C; ENL: 4.6; PA: 4; ENL + PA: 4).

3.4. Effects of HepG2 exposure to PA and/or ENL on the expression of the proteins involved in eicosanoids and prostanoids production and inflammatory process

Total expression of 15-LOX was significantly elevated in the HepG2 cells exposed to PA alone as well as PA with ENL, as compared to the control group (Fig. 3B; PA: +22.9%; ENL + PA: +28.3%). In the case of COX-2 expression we revealed a substantial increase in its level in all of the examined groups (Fig. 3A; ENL: +20.9%; PA: +22.9%; ENL + PA: +33.2%). In addition, we found an up-regulated TNFα expression in all of the examined groups (Fig. 3C; ENL: +16.6%, PA: +18.5%, ENL + PA: +21.5%). The changes in the PA + ENL-treated group were statistically significant as compared to the palmitate-treated group.

3.5. Effects of HepG2 exposure to PA and/or ENL on the expression of the proteins directly involved in the desaturation and elongation of fatty acids

SCD 1 expression remained unchanged in all of the examined groups (Fig. 4A). Nonetheless, we observed a substantial rise of elongase 3 (Elovl 3) expression in the groups treated with PA alone or PA and ENL simultaneously (Fig. 4B; PA: +23.5%; ENL + PA: +31.7%). The expression of elongase 6 (Elovl 6) was also elevated, but only after the exposure to PA and ENL (Fig. 4C; ENL + PA: + 30.7%).

4. Discussion

Recently, there is an increasing interest in the use of rich-polyphenol diet in the treatment of hepatic steatosis [22]. This is because many researchers believe that polyphenols and other phytochemicals interact with lipid metabolism, thus affecting tissue lipogenesis and fatty acid composition [15]. In our studies, we focused on enterolactone's - a phytoestrogen produced by the intestinal microflora from dietary precursors - properties [23]. Although numerous studies have confirmed its potential involvement in lipid metabolism, it is still unclear if ENL has lipogenic or antilipogenic character and to what extent it affects fatty acids composition in particular lipid fractions [24,25]. In the present study we showed that palmitate is the main fatty acid accumulated in all of the examined lipids (FFA, DAG, TAG) in response to its high availability in the incubation media. This is consistent with the previous studies conducted in mice fed with high-carbohydrate or high-fat diet and it confirms that fatty acids profiles in the liver are determined by the composition of a diet [25]. In hepatocytes, the excess of intracellular palmitate is converted to other fatty acids in numerous metabolic pathways. One of them is the elongation process catalyzed by the elongase enzymes (Elovl 3 and Elovl 6) [26]. Our results clearly demonstrated, especially in TAG fraction, that the elongation of palmitic acid (16:0) to stearic acid (18:0) and then to

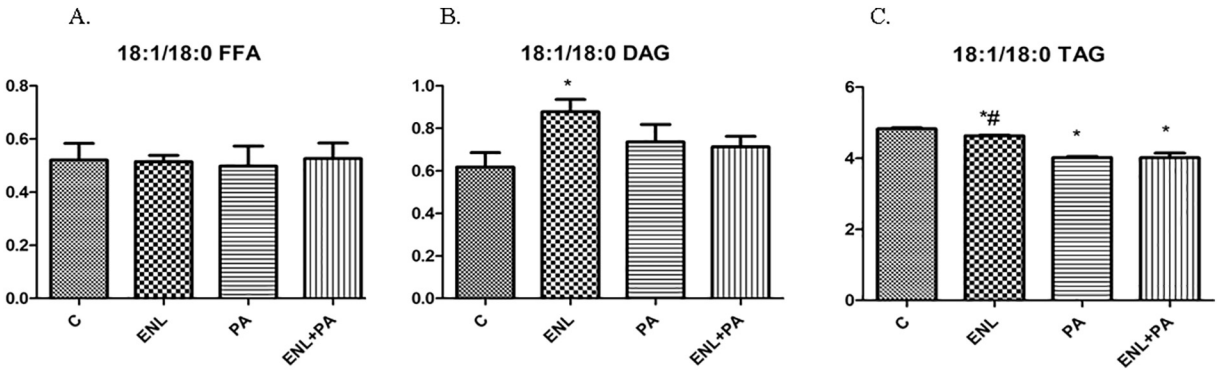

Fig. 2. The desaturation index in the free fatty acids (A), diacylglycerols (B) and triacylglycerols (C) in HepG2 cells.
The desaturation index is expressed as the ratio of unsaturated to saturated fatty acids. The cells were incubated with enterolactone (50 μM) alone or combined with palmitic acid (0.5 mM) for 16 h as it was described in details in Materials and methods section. The fatty acid content in HepG2 cells was measured by GLC method. The data are expressed as the mean ± S.D. and are based on six independent determinations. *P < 0.05 significant difference vs control group; ** 0.05 < P < 0.01 significant difference vs control group; #P < 0.05 significant difference vs palmitate-treated group; C - Control, PA - Palmitate, ENL - Enterolactone, ENL + PA - Enterolactone + Palmitate.

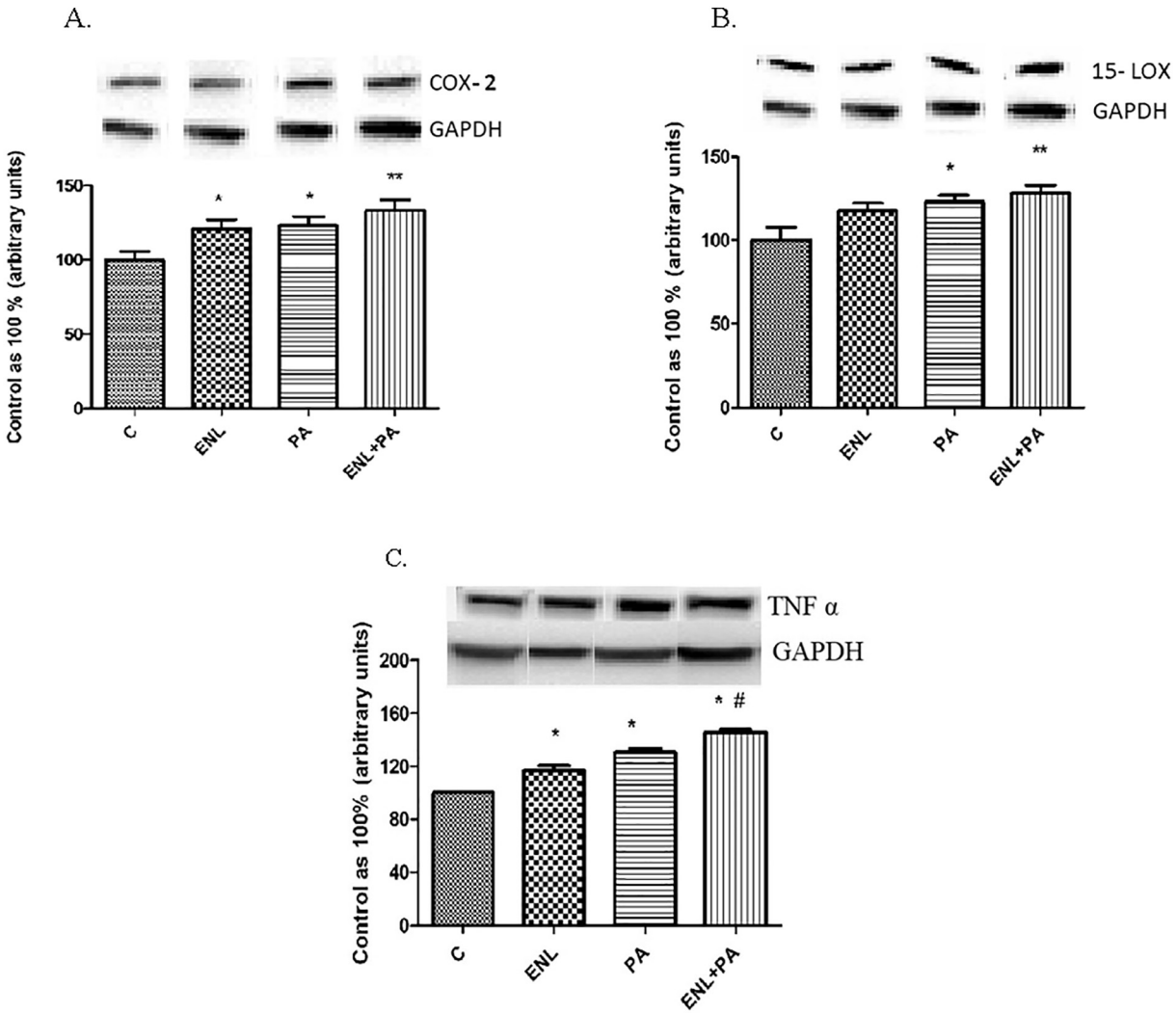

Fig. 3. The expression of proteins involved in eicosanoids and prostanoids production: COX-2 (A), 15-LOX (B) and inflammatory process: TNFα (C).
The cells were incubated with enterolactone (50 μM) alone or combined with palmitic acid (0.5 mM) for 16 h as it was described in details in Materials and methods section. The protein expression in HepG2 cells was measured using Western blot method. The data are expressed as the mean ± S.D. and are based on six independent determinations. *P < 0.05 significant difference vs control group, ** 0.05 < P < 0.01 significant difference vs control group; #P < 0.05 significant difference vs palmitate-treated group; C - Control, PA - Palmitate, ENL - Enterolactone, ENL + PA - Enterolactone + Palmitate.

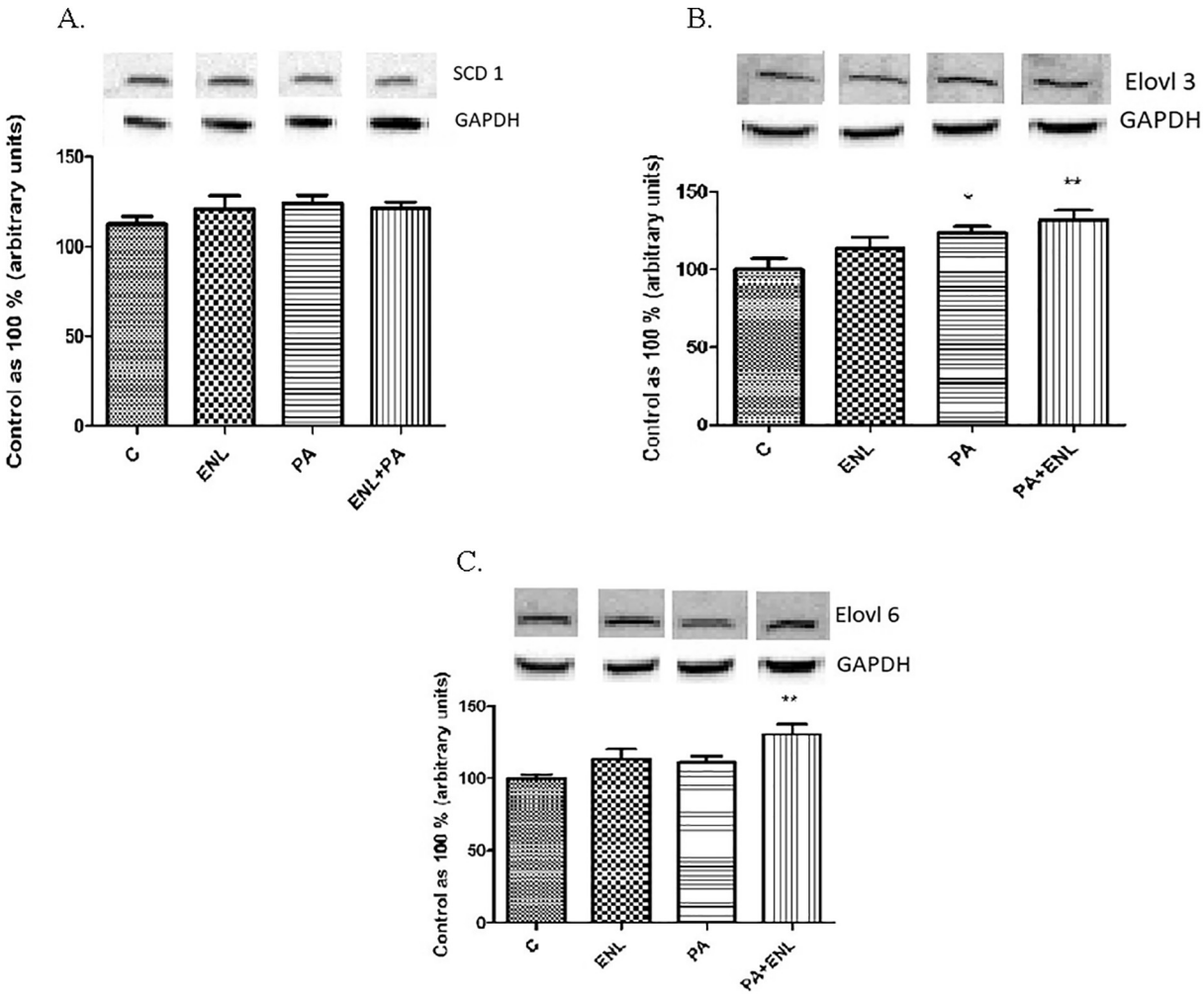

Fig. 4. The expression of proteins involved in desaturation: SCD 1 (A) and elongation of fatty acids: Elovl 3 (B), Elovl 6 (C).
The cells were incubated with enterolactone (50 μM) alone or combined with palmitic acid (0.5 mM) for 16 h as it was described in details in Materials and methods section. The protein expression in HepG2 cells was measured using Western blot method. The data are expressed as the mean ± S.D. and are based on six independent determinations. *P < 0.05 significant difference vs control group, ** 0.05 < P < 0.01 significant difference vs control group; #P < 0.05 significant difference vs palmitate-treated group; C - Control, PA - Palmitate, ENL - Enterolactone, ENL + PA - Enterolactone + Palmitate.

arachidic acid (20:0) was intensified in all of the experimental groups. Previous studies concerning the influence of other polyphenols (resveratrol) revealed that it affected Elovl expression in brown adipocytes [27]. However, to the best of our knowledge, this is the first time when the effect of ENL on the FA elongation process in hepatocytes has been reported. We found an increase in the protein expressions of Elovl 3 and Elovl 6 in the ENL + PA-treated group, which additionally corroborates the elongation phenomenon. Interestingly, enterolactone alone did not

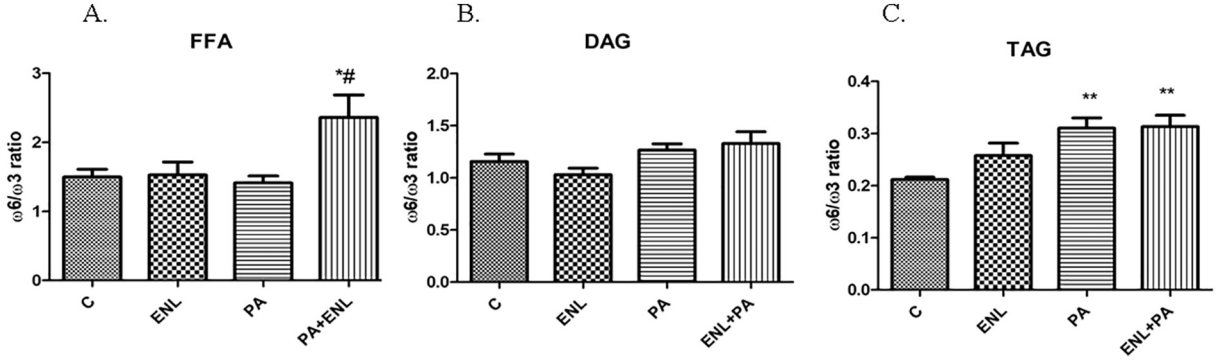

Fig. 5. The ω-6/ω-3 fatty acids ratio in the free fatty acids (A), diacylglycerols (B), triacylglycerols (C).
The cells were incubated with enterolactone (50 μM) alone or combined with palmitic acid (0.5 mM) for 16 h as it was described in details in Materials and methods section. Total lipid content was measured by GLC method. The data are expressed as the mean ± S.D. and are based on six independent determinations. *P < 0.05 significant difference vs control group; ** 0.05 < P < 0.01 significant difference vs control group; #P < 0.05 significant difference vs palmitate-treated group; C - Control, PA - Palmitate, ENL - Enterolactone, ENL + PA - Enterolactone + Palmitate.

influence the expression of the elongase proteins. Therefore, it is possible that it has an additive effect on the elongation process only during increased lipids availability. Furthermore, intracellular palmitic acid metabolism includes also its desaturation process into palmitoleic (16:1) or oleic acid (18:1) by SCD 1 [28]. It has been proven that polyphenols reduce the expression of SCD 1 [29]. Nonetheless, in our study we did not notice any changes in this protein expression after ENL supplementation. Despite the lack of changes in SCD1 protein expression, the desaturation process occurred in the group of cells incubated with ENL, as manifested by the accumulation of desaturation products, i.e. 18:1, 18:2, 18:3, and 20:4 in the free fatty acids and triacylglycerols, as well as 18:1 and 18:3 in the diacylglycerols. Therefore, to more carefully address this issue we evaluated FFA's, DAG's and TAG's desaturation index. The index is a well-recognized, simple and reliable indicator of SCD 1 activity [30]. Based on the increased DAG's desaturation index in the cells exposed to enterolactone we can assume that this polyphenol enhances the bioavailability of unsaturated fatty acids, that, on the other hand, are essential substrates used for the synthesis of phospholipids, cholesterol esters or other lipid fractions such as TAG. However, in TAG fraction decreased desaturation ratio after enterolactone treatment showed that lack of 16:1 and 18:1 accumulation decreased the possibility of fatty acid redistribution among already deposited lipid fractions. Moreover, the observed lack of changes in SCD 1 expression suggests that the enzyme activity may be insufficient to counteract high levels of palmitic acid in the incubation media. It should also be mentioned that the diversity of synthesis pathways, that results from changes in the expression of both desaturases and elongases, may alter intracellular levels of different fatty acids pools causing metabolic syndrome or hepatic steatosis [31,32]. According to Matsuzaka et al., Elovl 6 plays an important role in the activation of palmitate-induced inflammation in hepatocytes and aggravation of the inflammatory process in the patients with NASH [14]. Paradoxically, the researchers found that the intermediate products of palmitate metabolism produced by Elovl 6 action intensified proinflammatory cytokines release more than palmitate itself. Thus, it is likely that the augmentation of elongation by ENL reflects its pro-inflammatory nature. It is known that the process of desaturation may result in the formation of mono- and polyunsaturated fatty acids (PUFA). Two main types of PUFA are ω-3 and ω-6 fatty acids [33]. Both types participate in the regulation of inflammation and a balance between them determines cellular homeostasis [34]. Linoleic acid (LA), a representative of ω-6 fatty acids, is a precursor of arachidonic acid (AA). AA is a substrate for 15 lipoxygenase (15-LOX) and cyclooxygenase 2 (COX-2) production.

They catalyze the insertion of oxygen molecules into PUFA, thus contributing to the production of proinflammatory mediators [35]. Little is known about the possible polyphenols' effects on 15-LOX and COX-2 activities in hepatocytes [36]. Herein, we found the ENL aggravated COX-2 protein expression in HepG2 cells, which could explain the observed proinflammatory features of enterolactone. Furthermore, the rise of 15-LOX and COX-2 proteins expressions in ENL + PA-treated group is compatible with the detected accumulation of AA in TAG and FFA fractions. There are few somewhat contradictory data on the role of TAG in metabolic diseases development. Listenberger et al. found that an excessive accumulation of this lipid fraction does not pose threats to liver functioning [37]. In contrast, a report from Yamaguchi and coworkers showed that an undue TAG accretion exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis [38]. The data presented herein suggest that ENL alters fatty acid composition of triacylglycerol, thus making it less safe for hepatocytes. Moreover, all of the investigated lipid fractions (FFA, TAG, DAG) were characterized by increased ALA levels. ALA is converted through elongation and desaturation processes to eicosapentaenoic (20:6 ω-3) acid (EPA). EPA, on the other hand, may be oxidized by COX and LOX enzymes and transformed to active metabolites, i.e. molecules with anti-inflammatory and mediators pro-resolving properties [39]. To date, detailed animal and human studies have demonstrated that ω-3 PUFA also reduce the inflammatory phenotype via modulation of multiple molecular pathways. Previous studies showed that polyphenols aggravate intracellular ω-3 fatty acids accumulation. For instance, Caro et al. investigated that wine lees administration causes the exacerbation of TAG's EPA level in zebrafish embryos [40]. However, we did not find any changes in intracellular EPA concentration in any of the examined groups. Based on above, we may suspect that ENL acts as a proinflammatory agent since it seems to favor ALA over EPA accumulation in the liver cells. Moreover, to better determine its (ENL) postulated inflammatory effects we examined the ω-6/ω-3 fatty acids ratio. A handful of research showed excessive levels of ω-6 PUFA and their oxidized derivatives in intrahepatic fat in steatohepatitis [41]. Moreover high ω-6/ω-3 ratio has been repeatedly described as a factor that exacerbates inflammation in the development of NASH [11,42]. We observed a shift in the balance between ω-6 and ω-3 to ω-6 PUFA in the ENL + PA treated group in both FFA and TAG. Therefore, we suspect that ENL may provoke a redistribution of fatty acids towards proinflammatory processes but only in lipid overload state. Furthermore, all the described changes in lipid metabolism, caused by enterolactone supplementation, predispose to inflammation development, as confirmed by the increased protein expression of TNFα in HepG2 cells. Moreover, the addition of this polyphenol to palmitate intensified this effect even further. Surprisingly, our investigation is inconsistent with in vitro studies conducted in human peripheral blood lymphocytes and human monocyte cell line (THP-1) showing that ENL inhibited transcription and translation of TNFα in immune cells [43]. The only possible explanation of this discrepancy is the fact that liver cells respond differently to ENL treatment because of being derived from more metabolically complex tissue, namely liver. However, the precise mechanism of enterolactone action on TNFα pathway in hepatocytes still needs to be determined.

There are limitations of the study the foremost of which is using tumor hepatic cell line instead of healthy primary hepatocytes. This may be important because the ENL treatment may exert different effects on malignant cells. On the other hand, HepG2 cells are widely used cell model expressing many features of primary hepatocytes. However, in vivo studies must confirm ENL influence on liver lipid metabolism and proinflammatory effects.

5. Conclusions

The obtained results clearly indicate that ENL considerably modifies fatty acids compositions in the investigated lipid fractions (FFA, TAG, DAG). Moreover, we showed that the exposition to this polyphenol leads to fatty acids elongation, but not their desaturation in the hepatocytes. Importantly, this phytochemical shifted the ω-6/ω-3 balance towards ω-6, although only in a lipid overload state. Furthermore, based on the observed increase in COX-2 and TNFα expression, we may postulate that this polyphenol exerts proinflammatory effects.

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Conflict of interest

All authors declare that they have no competing interests.

Author contributions

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

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