THE INFLUENCE OF POLYUNSATURATED FATTY ACIDS ON CHOLESTEROL HOMEOSTASIS

Valová M., Komprda T., Zorníková G., Knoll A., Vykoukalová Z., Rozíková V., Škultéty O., Krobot R.

Department of Food Technology, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic

E-mail: xvalov11@node.mendelu.cz

ABSTRACT

The aim of this work was to validate the hypothesis of a positive influence of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) on the proportion of plasmatic lipids. These polyunsaturated fatty acids can decrease the plasma cholesterol due to the increase in expression of the gene Insig-1 together with the decrease in expression of the genes coding HMG-CoA-R and LDL-R. Hypothesis had been tested on experimental animals (*Rattus norvegicus*) that were fed with a standard feeding mixture, with the addition of 3 % fish oil (salmon oil). The expression of the gene Insig-1 in animals livers that had been fed with the feed with the additon of salmon oil was 730 % ($P < 0.05$) compared to the control. However, on the contrary to the hypothesis, the expression of the gene HMG-CoA-R was 165 % ($P > 0.05$) and the expression of the gene for LDL-R was 210 % ($P > 0.05$) compared to the control. Nevertheless, it was proved ($P < 0.05$) that the fish oil used for the diet of the experimental rats decreased the plasma cholesterol by 10 % (from the initial value 1.19 $mmol.l^{-1}$ to the finally value 1.07 $mmol.l^{-1}$). The conclusion of experiment was confirmation of the presumptive hypothesis, but it was also found the fact that the metabolism of lipids in plasma is influenced by another unexpected mechanism.

Key words: docosahexaenoic acid, eicosapentaenoic acid, real-time PCR, expression, cholesterol

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INTRODUCTION

The aim of my final thesis was to confirm the hypothesis of a positive effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the plasma lipids concentration and how they affect the expression of target genes. The effect of EPA/DHA on blood lipid level is based on the action of these PUFAs n-3 as ligands of the various isoforms of peroxisome proliferator-activated receptor (PPAR), and on modulation of the signaling pathway of the transcription factor sterol response element-binding protein, SREBP. Regarding plasma triacylglycerol (TAG) levels, the protective effect of EPA/DHA is sufficiently explained: PPARα activation and inhibition of the SREBP-1 signaling pathway stimulates fatty acid (FA) β-oxidation and inhibits FA synthesis with the final result of decreased serum TAG (Jump, 2008). The situation is much less clear as far as cholesterol is concerned (Komprda, 2012). A principal transcription factor binding the promoter region of the genes coding for proteins controlling cholesterol homeostasis (3-hydroxy-3-methyl-glutaryl-CoA reductase, HMG-CoA-R; low density lipoprotein receptor, LDL-R) is SREBP-2 (Nakamura et al., 2004). SREBP-2 release from the endoplasmic reticulum and consequently its activation in the Golgi apparatus is affected by an amount of protein INSIG (insulin-induced gene), product of the Insig gene (Sato, 2010). SREBP-2 is not directly ligated by EPA/DHA; a relationship, still not unequivocally explained, between PPARα ligation and SREBP-2 activation is presumed (Luci et al., 2007). König et al. (2007) suggested presence of a PPAR-responsive sequence in the Insig-1 gene promoter.

MATERIAL AND METHODS

Adult male rats of the laboratory strain Wistar Albino were used. Rats were randomly divided into three groups with ten animals each. The basic feed mixture, pelletized complete chow for mice and rats was used. The experimental diet was formed by adding of 3 % of salmon oil to the chow (F). The chow with 3 % of palm oil (P) was served as a negative control with a presumed cholesterol-increasing effect. The chow with an adequate amount of maize starch to render the diet isocaloric was designated as a control (C). At the end of the experiment lasting 48 days, blood samples were collected by cardiac puncture under anesthesia with isoflurane into the heparin-coated test tubes and centrifuged at 200 x g for 10 min at 4 °C to obtain plasma. Liver was removed and RNA was isolated immediately from an aliquot of 1 g.

Total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen). One μg of the isolated RNA was reverse transcribed using Omniscript RT Kit (Qiagen) and oligo-dT primers. Obtained cDNA was used for quantitative PCR with specific primers for the rat Insig1 gene (fw TCTTCCCGGACGAGGTGATAG, rev AGCTGCACATTATTGGCGAAAT), Hmgcr gene (fw AAGGGGCGTGCAAAGACAATC, rev ACACGGCACGGAAAGAACCATAG T), Ldlr gene (fw GGACAAGTCGGACGAGGAGAA, rev AGCTGATGCACTCCCCACTGT), PPARα gene (fw GCCTTGTCCCCACATATTCG, rev AGAGGAGAGTTCCGGAAG), SREBP-2 gene (fw ATCCGCCCACACTCACGCTCCTC, rev GGCCGCATCCCTCGCACTG) and housekeeping gene Actb (fw AGAGGGAAATCGTGCGTGAC, rev GTTTCATGGATGCCACAG GATT).

The reaction mixture was as follows: 1 μl of cDNA; 0,2 μl of AmpErase® Uracyl N-glycosylase (Applied Biosystems); 10 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems); 0,2 μl of each primer; 8,4 μl of H_2O. All analyses were carried out on the 7500 Real-Time PCR System (Applied Biosystems) under the following conditions: 2 min of UNG incubation at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at specific annealing temperature that was either 65 °C (expression of the Insig 1 and Ldlr gene) or 60 °C (expression of both remaining genes) and 30 s at 60°C. Effectivity of each reverse transcription reaction was calculated based on the standard curve method using decimal dilution of the input cDNA. The specificity of each PCR fragment was

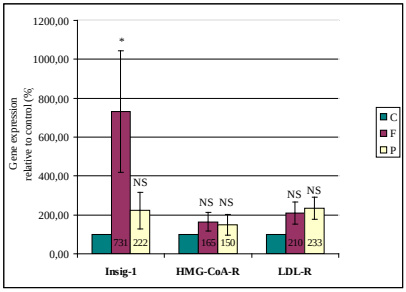
verified by the sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

Total plasma cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and TAG were determined by the enzymatic-colorimetric method using an automated chemical analyser BS-200 (Mindray, China) and commercial kits (Greiner Diagnostic GmbH, Germany).

RESULT AND DISCUSSION

Relative expression of the genes coding PPARα and SREBP-2 in the liver of the FO-fed rats was 47 % and 57 % as compared to the control. Due to the great variability of the CT values, the differences were insignificant (P>0.05). Relative expression of the Insig-1 gene in the liver of rats fed the diet with 3 % of fish oil was 730 % of expression of this gene in the control rats (P<0.05); however, an assumption that an over-expression of the Insig-1 gene leads to down-regulation of the HMG-CoA-R gene and LDL-R gene, respectively was not confirmed (Figure 1). Expression of the HMG-CoA-R gene and LDL-R gene in the liver of the FO-fed rats was 165 % and 210 % relative to the expression of these genes in the liver of the control rats; again, the differences were insignificant (P>0.05) due to the great variability of the CT values within each tested group of rats.

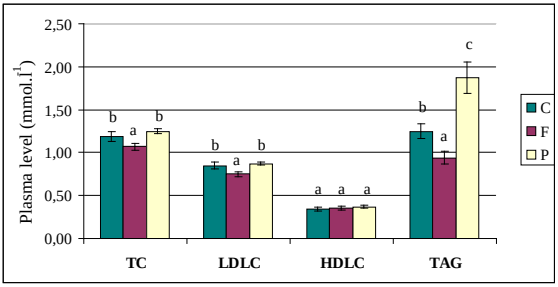
Plasma levels of total cholesterol and its fractions are shown in Figure 2. FO in the diet decreased (P<0.05) total plasma cholesterol and LDL cholesterol in rats by 10 % and 12 %, respectively as compared to the control diet. HDL-C was not changed by the dietary intervention. Plasma TC level of the FO-fed rats in the present experiment (1.07 mmol.l^{-1}; Figure 2) is approximately in the middle of the range of the results of similar experiments (FO-fed rats; all data recalculated to mmol.l^{-1}): 3.22 (Lu et al., 2011) → 2.45 (Ferramosca et al., 2012) → 2.36 (Xiao et al., 2012) → 2.04 (Campioli et al., 2012) → 1.01 (Yamazaki et al., 2011) → 0.98 (Takahashi, 2011) → 0.54 (Popovic' et al., 2011). The same is regarding plasma LDL-C in the present experiment (0.75 mmol.l^{-1}; Figure 2): the results of the similar experiments with the FO-fed rats are between 1.84 mmol.l^{-1} (Xiao et al., 2012) and 0.23 mmol.l^{-1} (Popovic' et al., 2011), the value reported by Campioli et al., 2012 (0.81 mmol.l^{-1}) being very similar to our data. Published data regarding HDL-C also vary conspicuously between 1.11 mmol.l^{-1} (Ferramosca et al., 2012) and 0.17 mmol.l^{-1} (Popovic' et al., 2011), with the level found in the present experiment (0.35 mmol.l^{-1}) approximately in the middle.



C – rats fed the control diet (n = 10); F – rats fed the control diet with 3 % of fish oil (n = 10); P – rats fed the control diet with 3 % of palm oil (n = 10); Insig-1 – insulin-induced gene-1; HMG-CoA-R – 3-hydroxy-3-methyl-glutaryl-CoA reduktase; LDL-R – low density lipoprotein receptor; * – amount of mRNA in the sample differed from the control (P<0,05); NS – amount of mRNA in the sample did not differ from the control (P>0,05)

Figure 1. Expression of the genes presumably controlling cholesterol homeostasis.

In summary, the results of experiments evaluating an effect of FO on HDL-C in rodents are ambiguous. Both Campioli et al. (2012) and Yamazaki et al. (2011) did not find a difference in plasma HDL-C in the FO-fed and the control rats, similarly to our data. On the other hand, Popovic' et al. (2011) and Xiao et al. (2012) reported an increase of this marker in the rat plasma after FO intake. Contrary to the above-mentioned results, Takahashi (2011) found a decrease of this parameter from 1.56 mmol.l^{-1} (palm oil-fed rats) to 0.58 mmol.l^{-1} (FO group). Similar conclusions reported also Kamisako et al. (2012) in mice fed a diet with FO in comparison with soybean oil-fed control: FO decreased plasma HDL-C from 1.50 to 0.56 mmol.l^{-1}.



TC – total cholesterol; HDLC – high density lipoprotein cholesterol; LDLC – low density lipoprotein cholesterol; a, b, c – means with different letters within a given trait differ at P<0.05

Figure 2. Plasma cholesterol and triacylglycerols (TAG) of rats fed the control diet (C) and the control diet with either 3 % of fish oil (F) or 3 % of palm oil (P), respectively (n = 10);

CONCLUSIONS

In this experiment following hypotheses were tested. EPA and DHA can increase expression of the Insig-1 gene, which leads to suppression of the Hmgcr gene and Ldlr gene with a consequence of decreased plasma cholesterol. We were able to confirm, however, only the first and last point of this hypothesis. Therefore, it can be concluded that the cholesterol lowering effect of fish oil is at least partly based on mechanisms other than tested here. Nevertheless, it was proved ($P < 0.05$) that the fish oil used for the diet of the experimental rats decreased the plasma cholesterol by 10 %. Deviation in hypothesis we assumed may be caused by the fact that the experimental animals were fed by feed mixture with the addition of salmon oil, but they were not fed by feed mixture with the addition of pure active substance (EPA and DHA). Another reason could be also that dose of EPA and DHA was too low. Finally, the reason could be that the metabolism of lipids in the blood is influenced by a different mechanism than we expected. This issue requires further study and experiments with a modified methodology.

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