EFFECT OF DIETARY FISH OIL ON EXPRESSION OF LIVER GENES CONTROLLING CHOLESTEROL HOMEOSTASIS: COMPARISON OF TWO ANIMAL MODELS

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Abstract: The aim of this study was to compare the effect of the fish oil, respectively its main component, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the expression of genes PPARα, SREBP-2, Insig-1, Hmgcr and Ldlr which control cholesterol homeostasis in the liver of rats (Wistar Albino; n = 32) and pigs (Large White x Landrace; n = 32). Rats and pigs were randomly assigned into two groups of 16 animals and fed ten weeks by the diet with either 2.5% of fish oil (group F; source of eicosapentaenoic and docosahexaenoic acid, EPA+DHA) or 2.5% of palm oil (group P; high content of saturated fatty acids; control group). Dietary fish oil relative to palm oil increased PPARα and SREBP-2 gene expression much strongly (P<0.01) in the pig liver in comparison with the rat liver, but expression of Insig-1 and Hmgcr genes in the liver of the F-pigs relative to the expression of these genes in the liver of the P-pigs was substantially lower (P<0.01 and P<0.05, respectively) as compared to rats.

Key Words: Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), Gene expression, Cholesterol homeostasis, Sus scrofa, Rattus norvegicus

INTRODUCTION

This study focuses on the effect of n-3 polyunsaturated fatty acids, respectively eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the expression of liver genes which control cholesterol homeostasis. Assumed, that cholesterol is situated in the hepatocytes. The liver is the central organ of cholesterol homeostasis.

Polyunsaturated fatty acids (EPA and DHA) significantly affect gene transcription by regulating the activity of a number of transcription factors, including nuclear receptors such as PPARs (peroxisome proliferator activated receptor), or a group of transcription factors SREBP (Afman et al. 2012). Expression of the genes coding for the key proteins controlling cholesterol homeostasis, hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA-R) and low-density lipoprotein receptor (LDL-R), is stimulated by the transcription factor SREBP-2, whose activation is affected by the INSIG protein (insulin-induced gene), product of the Insig gene (Sato 2010). Moreover, SREBP-2 activation is presumably related to PPARα ligation by EPA/DHA (Luci et al. 2007).

In vivo studies are usually carried out on rodents. However, rodents (in this context “proliferating” species) are not ideal models for humans (“non-proliferating” species) opposed to pigs which are “non-proliferating” species as humans.

The objective of the present study was to compare two animal models, rat and pig, regarding an effect of fish oil (the most common source of EPA+DHA) on expression in the liver of the genes presumably affecting cholesterol homeostasis. An intention was to carry out an experiment as similar to usual human conditions as possible and to use dietary EPA+DHA (fish oil) in the amount realistically achievable in human nutrition. On the other hand, palm oil, containing a high percentage of saturated palmitic acid, and which is currently included in a broad spectrum of foods, can be mentioned in this context as a negative example.
In the present study was tested the hypothesis (Chatterjee et al. 2009; König et al. 2007) that PPARα activation by EPA/DHA increases the expression of the Insig-1 gene leading to the retaining of SREBP-2 precursor protein in endoplasmic reticulum, to the decrease of SREBP-2 active form and consequently to the decrease of Hmgcr and Ldlr gene expression. Therefore the expression of the genes of two transcription factors (PPARα and SREBP-2) and three subordinate genes (Insig-1, Hmgcr and Ldlr) was measured.

**MATERIAL AND METHODS**

**Animals, dietary interventions, analyzed tissues**

As a model animals for this study were used thirty-two male rats (laboratory strain Wistar Albin; Meditox Konárovice, Czech Republic) and thirty-two pigs of both sexes (16 females, 16 males; Large White x Landrace; Bioprodukt Knapovec a.s., Ústí nad Orlicí, Czech Republic). The rats were at the age of eight weeks with the mean live weight of 312 ± 23 g and pigs were at the age of eight weeks with the mean live weight of 25.5 ± 1.15 kg. The animals were divided into two groups (experimental and control) of 16 individuals (the same number of males and females was in each group in the case of pigs). The experimental group was fed for ten weeks with the basic feed mixture with 2.5% of fish oil (F) and the control group was fed with the basic feed mixture with 2.5% of palm oil (P). The P-diet was used as a control in order to keep the diet not only isocaloric but also iso-lipidic within the given animal species. The rats were fed daily ad libitum and pigs were fed twice daily (7 a.m. and 2 p.m.). The animals were weighed in weekly intervals. After the experiment, the rats were sacrificed using inhalation anesthetics Isoflurane and the pigs were anesthetized by the intramuscular application of the TKX mixture (12.5 mg/ml of ketamine, 12.5 mg/ml of xylazine, 12.5 mg/ml of tiletamine, 12.5 mg/ml of zolazepam) in the total volume of 0.2 ml/kg of the live weight and sacrificed by bleeding. Total RNA was immediately isolated from liver aliquots (30 and 50 mg in rats and pigs).

**Quantification of the gene expression in the liver**

Total RNA was isolated from the liver using RNase® Mini Kit (Qiagen GmbH, Hilden, Germany); concentration was measured on NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and isolated RNA was stored at -80 °C. One μg of the isolated RNA was reverse transcribed using M-MLV reverse transcriptase system (Invitrogen, Paisley, UK) and oligo-dT primers. Obtained cDNA was used for quantitative PCR with specific primers characterized in Table 1. Different housekeeping genes were used in the rat (Actb) and pig (TBP1) liver samples.

Quantitative PCR was carried out using the Nanodrop II liquid dispenser (Innovadyne Technologies, Rohnert Park, CA). RNA expression was quantified in triplicate reactions in a final volume of 3 μl in 384-well plates using QuantiTect SYBR Green PCR master mix following the manufacturer's recommendations (Qiagen, Hilden, Germany) on a LightCycler 480 (Roche Applied Science; https://www.roche.com under the following conditions: denaturation at 95 °C for 15 min and 45 amplification cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. The reaction mixture consisting of 0.5 μL of cDNA, 1.5 μL of SYBR® Green PCR Master Mix and 10 pmol of each couple of primers (Generi Biotech, Hradec Králové, Czech Republic) was used.

**Table 1** Primers used for quantitative PCR

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Species</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>Rat</td>
<td>GCCTTCTCCCACTATATTCG</td>
<td>AGAGGAGAGTCCCGGAAG</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>ATACCGCAGATCTGAGATGTCTC</td>
<td>ATAGCAGAAAGCGGTTTAC</td>
</tr>
<tr>
<td>SREBP2</td>
<td>Rat</td>
<td>CTGCCCTACCCCAAGGTTTCC</td>
<td>GAGCGCTGCTCTGAATAG</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>TCTTCCCCGAGCAGGTTATAG</td>
<td>AGTCCGCTCTCACATAG</td>
</tr>
<tr>
<td>Insig1</td>
<td>Rat</td>
<td>GAAAAATCCACCCTCTCTCGACTTGG</td>
<td>AAAAGACCAATGCCTTGGC</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>GAAAAATCCACCCTCTCTCGACTTGG</td>
<td>AAAAGACCAATGCCTTGGC</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>Rat</td>
<td>AAGGGGCGCTGCAAAGGAAATC</td>
<td>AACACGCAGCGAAAAAGCATTAT</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>CTGATGAGCCTGCGAGAGGAGAATG</td>
<td>CGAATTCGCAATGACATAG</td>
</tr>
<tr>
<td>Ldlr</td>
<td>Rat</td>
<td>AAACCCTGTAGCTACGAGAAGCCAC</td>
<td>AGGCTGACCCACCTGGATAG</td>
</tr>
<tr>
<td>Actb</td>
<td>Rat</td>
<td>AGAGGGAAATCCTGCGATGAC</td>
<td>GTTTCATGGATGCCCAGGATT</td>
</tr>
<tr>
<td>TBP1**</td>
<td>Pig</td>
<td>AACAGTTCCATTGTTAGCCGAGAAG</td>
<td>AGATGGTCCTCAAAGCCCTAG</td>
</tr>
</tbody>
</table>

Legend: * PPARα = peroxisome proliferator-activated receptor α; SREBP2 = sterol response element-binding protein 2; Insig1 = insulin-induced gene; Hmgcr = 3-hydroxy-3-methyl-glutaryl-CoA reductase; Ldlr = low-density lipoprotein receptor; Actb = β-actin; all designed; **TBP1 = TATA box-binding protein, reference: Nygard et al. 2007
Statistical evaluation

All data were statistically analyzed using STATISTICA 12 package (StatSoft, Tulsa, OK, USA). For testing the differences between animal species in the relative expression of the liver genes was used Wilcoxon signed-rank test.

RESULTS AND DISCUSSION

Live weight, daily weight gain

Daily weight gain of rats was 3.04 ± 0.14 g/day for F-diet and 3.32 ± 0.18 g/day for P-diet. Daily weight gain of pigs was 0.85 ± 0.05 kg/day for F-diet and 0.86 ± 0.04 kg/day for P-diet. The final weight of the F-rats was 524.8 ± 13 g and P-rats 544.4 ± 11 g and final weight of the F- and P-pigs was 83.64 ± 1.82 kg and 84.06 ± 3.35 kg. Despite a slight tendency (P = 0.12) of fish oil to decrease daily weight gain in rats, type of oil in the diet affected (P>0.05) neither daily weight gain nor the final weight in either tested animal species.

Liver genes expression rate

Substantial differences between the tested species in an extent of the relative expression of the genes presumably controlling cholesterol homeostasis are apparent from Figure 1. Dietary fish oil relative to palm oil increased \( \text{PPAR}\alpha \) gene expression much strongly (P<0.01) in the pig liver in comparison with the rat liver. The same result was obtain in SREBP-2 gene (P<0.05). On the other hand, the expression of \( \text{Insig-1} \) and \( \text{Hmgcr} \) in the liver of pigs fed the diet with fish oil relative to the expression of these genes in the liver of the palm oil-fed counterparts was substantially lower (P<0.01 and P<0.05, respectively) in comparison with rats. The only gene whose relative expression in the liver did not differ (P>0.05) between pigs and rats was \( \text{Ldlr} \).

In this study was measured the expression of the genes of two transcription factors (\( \text{PPAR}\alpha \) and SREBP-2) and three subordinate genes (\( \text{Insig-1} \), \( \text{Hmgcr} \) and \( \text{Ldlr} \)).

As far as \( \text{PPAR}\alpha \) activation is concerned, fish oil caused a substantially lower increase (P<0.01) of relative expression of \( \text{PPAR}\alpha \) gene in the rat liver in comparison with pigs. Our results agree with the data of Arai et al. (2009) and Cheon et al. (2005) who reported higher expression of \( \text{PPAR}\alpha \) in pigs than in rodents after the administration of \( \text{PPAR}\alpha \) ligands.

Regarding SREBP-2, we have measured the same results as \( \text{PPAR}\alpha \). Woo et al. (2005) mentioned that SREBP-2 is a weak transcription activator by itself and needs a presence of additional transcription factors.

Cheon et al. (2005) reported that \( \text{PPAR}\alpha \) activation does not influence SREBP-2 controlled transcription of genes involved in cholesterol homeostasis. According to Luci et al. (2007), an effect of
PPARα activation on SREBP-2-dependent cholesterol synthesis may be different between various species.

The hepatic Insig-1 gene was up-regulated in rats and pigs in the present study (349 and 132% of the control in rats and pigs). But in the study of König et al. (2007), the suggested signal pathway via PPARα activation was confirmed only in pigs (up-regulation of the PPARα gene to 221% of the control; contrary to rats, where the gene was down-regulated to 52% of the control).

Despite the fact that pigs and rats differed in the expression of the Hmgcr gene but not in the expression of the Ldlr gene, both Hmgcr and Ldlr genes were up-regulated in the present experiment by the fish oil diet both in rats (196 and 113% of the control) and in pigs (134 and 123% of the control), which is contrary to the suggested signal pathway. However, this up-regulation of Hmgcr and Ldlr genes after administration of relatively low doses of fish oil (2.5%) confirms our previous results in rats consuming much higher fish oil doses (6% of the feed ration; Komprda et al. 2015).

CONCLUSION

The pigs as “non-proliferating” species are more ideal models for humans than rats (“proliferating” species) so we expected better results in pigs.

According to our hypothesis, dietary fish oil relative to palm oil increased PPARα and SREBP-2 gene expression much strongly (p < 0.01) in the pig liver in comparison with the rat liver. However, the expression of Insig-1 and Hmgcr genes in the liver of the F-pigs relative to the expression of these genes in the liver of the P-pigs was substantially lower (p < 0.01 and p < 0.05 respectively) as compared to rats.

Most of our results exactly match the results of other studies in the available literature. However, it would be appropriate to carry out further studies on this issue.

ACKNOWLEDGEMENTS

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The experiment was performed in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals against cruelty, the Amended Act No. 162/1993 Coll., and was approved by the “Commission to protect animals against cruelty” of the Mendel University in Brno and of the Ministry of Agriculture of the Czech Republic.

REFERENCES


