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LIPOXYGENASE ACTIVITY DURING THE CONDITION OF EXPERIMENTAL HYPERTRIACYLGLYCEROLAEMIA

LIPOXYGENÁZA V PODMIENKACH EXPERIMENTÁLNEJ HYPERTRIACYLGLYCEROLÉMIE

Original research article

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Abstract Animal lipoxygenases (EC 1.13.11.12, LOXs) convert arachidonic acid into biologically active compounds known as eicosanoids. Pro-inflammatory eicosanoids – leukotrienes – are involved in the progression of atherosclerosis. Our attention was focused on a connection between the activity of LOX and primary hypertriacylglycerolaemia, untreated or treated with fenofibrate. Our results show that LOX activity in the cytoplasm derived from the liver of hypertriacylglycerolaemic rats is significantly increased (pH 7.2; relative to the control group), which may be one reason for more rapid atherosclerosis progression in lipid metabolism disorders. The highest LOX activity in microsomes was observed in groups of rats treated with fenofibrate. It appears that fibrates indirectly promote association of LOXs to membranes. In fat fraction, no significant effect of hypertriacylglycerolaemia on the activity of LOX was found. Using gel electrophoresis, significantly different spectrum of proteins was discovered in the control samples and samples of hypertriacylglycerolaemic rats. It appears that in condition of lipid metabolism imbalance formation of proteins with low molecular weight (and possibly also the expression of LOX) is elevated.

Slovak abstract Živočíšne lipoxygenázy (EC 1.13.11.12, LOX) konvertujú kyselinu arachidónovú na biologicky aktívne zlúčeniny, známe ako eikozanoidy. Leukotriény - prozápalové eikozanoidy sú zapojené do progresie aterosklerózy. V predloženej práci sme sa zamerali na prepojenie medzi aktivitou LOX a primárnou hypertriacylglycerolémiou, neliečenou alebo liečenou fenofibrátom. Naše výsledky ukazujú, že aktivita LOX v cytoplazmatickej frakcii z pečene hypertriacylglycerolemických potkanov signifikantne stúpa (pH 7,2; v porovnaní s kontrolnou skupinou), čo môže byť jedným z dôvodov rýchlejšej progresie aterosklerózy pri ochoreniach lipidového metabolizmu. V mikrozomálnej frakcii sme najvyššiu aktivitu zaznamenali v skupine potkanov liečených fenofibrátom. Zdá sa, že fibráty nepriamo podporujú asociáciu LOX na membrány, a tým aj jej aktivitu. V tukovej frakcii nebol zaznamenaný žiadny výrazný efekt hypertriacylglycerolémie na aktivitu LOX. Použitím gélovej elektroforézy sme zistili, že spektrum proteínov v kontrolných skupinách sa značne líši od spektra vo vzorkách hypertriacylglycerolemických potkanov. Ukazuje sa, že pri poruchách lipidového metabolizmu sa zvyšuje tvorba proteínov s nízkou molekulovou hmotnosťou (a pravdepodobne aj expresia LOX).

Keywords *lipoxygenase; hypertriacylglycerolaemia; atherosclerosis; fibrates*

Kľúčové slová: *lipooxygenáza, hypertriglycerolémia, ateroskleróza, fibráty*

Abbreviations: AA, arachidonic acid; C, control group; CF, control group treated with fenofibrate; HDL, high-density lipoproteins; LDL, low-density lipoproteins; LTs, leukotrienes; LOX, lipoxygenase; VLDL, very low density lipoproteins; PPARα, peroxisome proliferator-activated receptors α; T, hypertriacylglycerolaemic rats; TF, hypertriacylglycerolaemic rats treated with fenofibrate.

1. Introduction

Lipoxygenases (LOXs, EC 1.13.11.12) belong to non-heme iron dioxygenases that catalyse stereo-specific oxidation of polyunsaturated fatty acids containing at least one 1-*cis*,4-*cis*-pentadiene system (Ivanov, 2010). Animal LOX converts arachidonic acid (AA) into hydroperoxyeicosatetraenoic acid and consequently to biologically active compounds eicosanoids, known as leukotrienes (LTs) (Bäck, 2009), lipoxins (Romano, 2006), hepoxilins (Pace-Asciak, 2009) and eoxins (Feltenmark et al., 2008). LTs are potent inflammatory mediators synthesised within the cardiovascular system through the 5-LOX pathway of AA metabolism. Recent experimental and genetic studies suggest a major role of LT signalling in progression of atherosclerosis and in its serious ischaemic complications such as acute coronary syndromes, stroke, aortic aneurysm and intimal hyperplasia (Riccioni et al., 2009).

Atherogenic dyslipidemia is a pathological condition characterised by an increase in elevated triacylglycerols (and increased very low density lipoprotein (VLDL) particle number), increased small LDL particles and decreased high-density lipoprotein cholesterol (HDL-C) (Grundy, 2004). Hypertriacylglycerolaemia

(high plasma level of triacylglycerols) is one of the factors causing endothelial vasodilatation in patients with hypercholesterolaemia. Triacylglycerols are implicated in the development of atherosclerotic lesions (Schneider et al., 2003).

Clinically, for the treatment of hypertriacylglycerolaemia fibrates are used. They activate PPARα-receptors in the liver. This activation induces the expression of the fatty acid transport protein and fatty acid translocase, which facilitates the transport of free fatty acids across the cell membrane. Activation of PPAR*α* also directly increases the transcription of enzymes of the peroxisomal β-oxidation pathway and *de novo* fatty acid synthesis by blocking enzymes such as acetyl-CoA carboxylase and fatty acid synthase (Schindler, 2007).

2. Materials and methods

Animals

Thirty male Wistar rats from Dobrá Voda in Slovak Republic, weighing between 175 and 265 g were housed in cages each separately. After 6-day adapting period on standard food, animals were divided into four groups and fed by different types of diet (Table 1).

Table 1. Animal groups fed by different types of diet (C – control group, T – hypertriacylglycerolaemic rats, TF – hypertriacylglycerolaemic rats treated with fenofibrate, CF – control group treated with fenofibrate)

Group	Number of animals in group	Standard food (g)	Pork fat (g)	Feno-fibrate (mg)
C	6	20	-	-
T	8	20	8	-
TF	8	20	8	15
CF	8	20	-	15

During the whole experiment, the animals were supplied with water *ad libidum* and underlied 12 hours circadian rhythm. Twenty-four hours before start of the experiment, the animals were allowed to starve. Experiment was carried out under good laboratory practice conditions. After 6 weeks, animals were sacrified by cervical dislocation after Avertin anaesthesia (i.p. 15.0 ml • kg^{-1}, 2.5% (v/v) solution). Organs (liver, epididymal adipose tissue) were rinsed in saline and immediately freezed in liquid nitrogen. Until analysis, the tissues were stored at -20°C.

Preparation of tissue homogenates

Livers were homogenised in a medium consisting of 0.25 M saccharose solution, adjusted by 0.15 mM Tris-HCl buffer to pH 7.4. Subcellular liver fractions (microsomes and cytoplasm) were obtained by differential centrifugation of 10–20% crude organ homogenate according to Cinti et al. (1972). White epididymal adipose tissue was homogenised in phosphate buffer, pH 7.4 with additament of Brij 96, to give final 1% (w/v) solution (0.2 g tissue/1.0 ml solution). After 15-min centrifugation (4°C, 2000 *g*) the postnuclear supernatant, which was finally tested, was obtained. Protein content in fractions was determined according to Bradford protocol (Bradford, 1976).

Enzyme activity

The activity of LOX was monitored as an increase in the absorbance at 234 nm, which reflects the formation of hydroperoxyoctadecadienoic acid. Linoleic acid (99%, Sigma; *cis*-9, *cis*-12-octadecadienic acid, $C_{18}H_{32}O_2$, Mr = 280.46, ε = 25,000 $mol^{-1} \cdot l \cdot cm^{-1}$) was used as a substrate prepared in solubilised state as described in Kemal et al. (1987). For the determination of LOX activity, a spectrophotometric assay was used on a spectrophotometer Perkin-Elmer UV/VIS LAMBDA 35. Incubation mixture contained 1400 µl of buffer (50 mM Tris–HCl buffer, pH 9.0 or 100 mM phosphate buffer, pH 7.2), 100 µl solution of linoleic acid prepared in solubilised state and 3 µl of enzyme protein. The enzyme activity was expressed in katal [kat] (1 kat = formation of 1 mol product in 1 s).

SDS–PAGE

For the determination of the apparent molecular masses of proteins, sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on a Bio-Rad Mini Protean II dual vertical slab gel electrophoresis cell (Bio-Rad Laboratories) according to Laemmli. (1970) by using 10% polyacrylamide gels. Proteins were stained with silver according to Nesterenko et al. (1994).

Statistical analysis

Values were expressed as mean ± SD. Experimental means were subjected to Student's *t*-test for two groups (C:T; T:F; C:CF). A probability value of $p<0.05$ was considered statistically significant.

3. Results and discussion

Effect of pH on LOX activity

The enzyme exhibited optimal activity at pH 7.2 and noticeably lower activity at pH 9.0 in all fractions and animal groups. Phosphate buffer with pH 7.2 creates optimal reaction condition for the catalytic activity of LOX.

LOX activity in cytoplasm

Compared with the other fraction, in cytoplasm the highest LOX activity in all animal groups was found. This could refer to the fact that LOX is primarily located in cytoplasm. The highest activity of LOX in this fraction was observed in groups of hypertriacylglycerolaemic rats (T) at pH 7,2. It is a significant increase in the enzymatic activity compared with the control group (C) ($p<0.001$) and it suggests that in lipid metabolism disorders the progression of atherosclerosis can be accelerated as a result of increased LOX activity. In the group of hypertriacylglycerolaemic rats treated with fenofibrate (F), the activity of LOX was decreased ($p<0.01$) and the lowest activity in the cytoplasm was observed in control groups treated with fenofibrate (CF). LOX activity in cytoplasm was reduced in response to fenofibrate treatment (Fig. 1).

LOX activity in microsomes

Compared with the cytoplasm and fat fractions, microsomes exhibited the lowest activity of LOX in all model groups. In this subcellular fraction, the highest level of enzyme activity was in groups treated with fenofibrate (F and CF) (p<0.001) (Fig. 2). A likely explanation for this phenomenon is the increased [Ca^{2+}] in the intracellular space, as one of the side effects of fibrates (Jiao and Zhao 2002). Bennett and Williams (1992) have demonstrated that ciprofibrate mobilises hepatic [Ca^{2+}]$_i$ via inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. These events may lead to an environment of elevated [Ca^{2+}]$_i$ and may serve to augment Ca^{2+}-dependent processes such as transfer of 5-LOX from the cytoplasm to the nuclear membrane resulting in its catalytic activity (Radmark et al., 2007).

LOX activity in fat fraction

In contrast with data from the literature that describes up-regulated 12/15-LOX mRNA in white epididymal adipocytes of high-fat fed mice (Chakrabarti et al., 2009), no significant correlation between hypertriacylglycerolaemia and elevated LOX activity in fat tissue was found. The highest LOX activity was showed in the control group treated with fenofibrate (CF) at pH 7.2 (p<0.001). Probably, it is a result of an abnormal adipocytes response of healthy rats to xenobiotic – fenofibrate (Fig. 3).

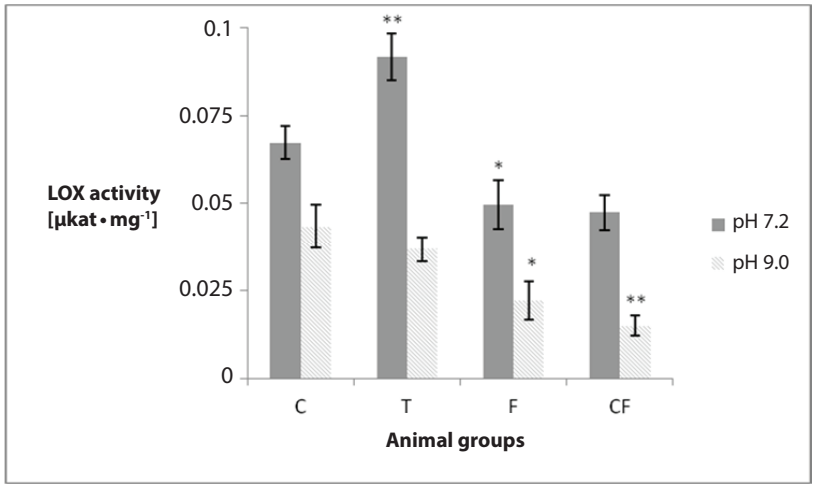

Figure 1. LOX activity in cytoplasm

Data are shown as means ± SD. Experimental means were subjected to Student's *t*-test for two groups (C:T; T:F; C:CF), ** p<0.001, * p<0.01. Non-marked results represent non-significant changes.

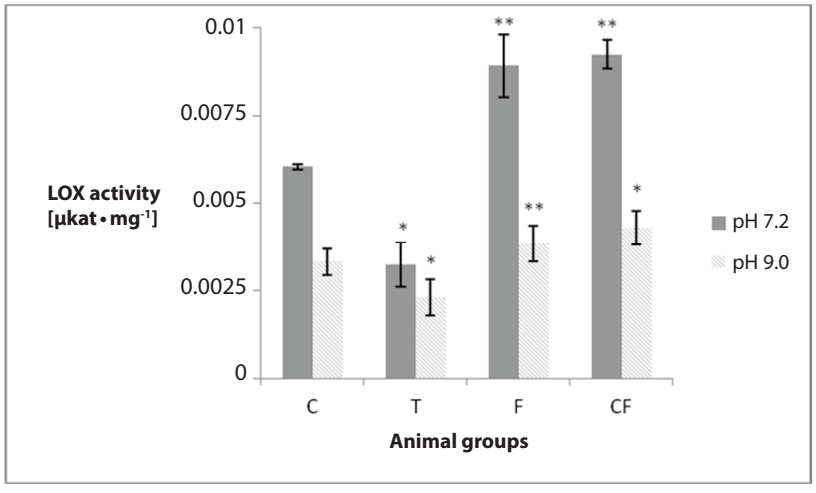

Figure 2. LOX activity in microsomes

Data are shown as means ± SD. Experimental means were subjected to Student's *t*-test for two groups (C:T; T:F; C:CF), ** p<0.001, * p<0.01. Non-marked results represent non-significant changes.

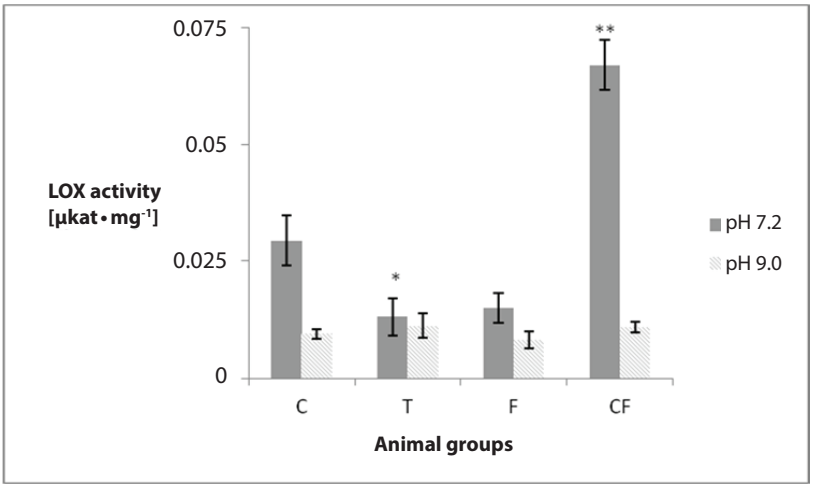

Figure 3. LOX activity in fat fraction

Data are shown as means ± SD. Experimental means were subjected to Student's *t*-test for two groups (C:T; T:F; C:CF), ** $p<0.001$, * $p<0.01$. Non-marked results represent non-significant changes.

Electrophoretic characterisation of proteins in samples with the highest LOX activity

For the electrophoretic characterisation, samples with the highest specific activity of LOX were chosen (cytoplasm fraction; pH 7.2; samples C3, T6, F3 and CF3). We found that the spectrum of proteins in the control samples (C3, CF3) is significantly different compared with samples with hypertriacylglycerolaemia (even in hypertriacylglycerolaemic rats treated with fenofibrate). While in control groups, there are three proteins in the range ± 80 kDa with apparent molecular masses 90.2; 82.1 and 74.7 kDa, in samples T6 and F3 a complex of proteins in the range 71.3–94.5 kDa is showed (Fig. 4).

Based on data from the literature, we conclude that the protein with a molecular mass of 74.7 kDa is probably 5-LOX isoform (Hogaboom et al., 1986). It suggests that lipid metabolism disorders lead to increase in formation of proteins with low molecular masses (and possibly also increase expression of LOX). It is interesting that fenofibrate had no effect on protein formation in cytoplasm, because a spectrum of proteins in control samples (C and CF) is similar.

4. Conclusion

In conclusion, we can say that LOX activity is significantly changed in conditions of hypertriacylglycerolaemia as well in its treatment with fenofibrate. Our results may bring a new insight into traditional treatment of atherosclerosis and lipid metabolism disorders.

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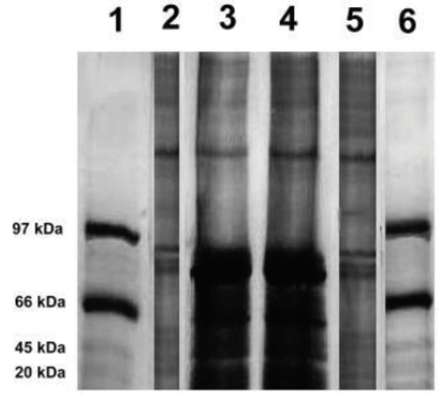

Figure 4. Electrophoretic characterisation of proteins in samples with the highest LOX activity

Band 1: LMW-standard (5 μl), **2:** K3 (7 μl), **3:** T6 (7 μl), **4:** F3 (7 μl), **5:** KF3 (7 μl), **6:** LMW-standard (5 μl)

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