

Paraoxonases gene expression and distribution in rats organs treated with atherogenic diet and atorvastatin therapy

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Paraoxonases isoenzymes, PON1, PON2 and PON3, have important antioxidative and anti-inflammatory properties in blood and cells. They prevent oxidation of low and high density lipoprotein particles, foam cells formation and development of atherosclerosis. The authors investigated effects of high fat diet and atorvastatin therapy on paraoxonases gene expression levels and distribution in different rat organs. Liver, white adipose tissue (WAT) and aorta were taken from young male Wistar rats that were fed with normal diet (ND), atherogenic diet (AD) and atherogenic diet with 1.14 mg of atorvastatin per kg (ADA). Messenger RNA (mRNA) relative levels of paraoxonase 1 (PON1), paraoxonase 2 (PON2) and paraoxonase 3 (PON3) were measured in rat organs using real-time polymerase chain reaction (PCR). PON1 mRNA expression levels were down-regulated in ADA compared to AD group. ND group had significantly lower PON2 mRNA expression than AD group. PON1 mRNA expression levels were higher in liver than in aorta in group of rats on ND, AD and ADA. PON2 mRNA expression was higher in WAT than in aorta only in ADA group of rats. PON2 and PON3 were significantly higher than PON1 in aorta of rats on each ND, AD or ADA.

Keywords: Paraoxonase, gene expression, atorvastatin, atherogenic diet, rat

Introduction

The paraoxonase (PON) multigene family comprises of three members, PON1, PON2 and PON3. PON1 and PON3 are expressed mostly in the liver and secreted into circulation where bound to plasma high-density lipoproteins (HDL)^{1,2}. PON2 is intracellular protein expressed in many tissues but not detected in plasma³. Opposite to human studies, data showed that mouse PON3 was not found in blood or HDL assuming it coexisted with PON2 in cells⁴. PON1, suggested by Mackness⁵, might have part in lipid metabolism and protect against atherosclerosis. PON1 and PON3 prevent the oxidation of HDL and low-density lipoproteins (LDL) where PON2 reduces intracellular oxidative stress (OS)^{1,3,6}. PON1, PON2 and PON3 have also anti-inflammatory properties.⁷ They share similarity in physiological properties but have different gene expression profiles^{3,8-9} which might be modulated by life style changes and medical treatment¹⁰⁻¹². PONs possess lactonase activity¹³ and

may have a role in the metabolism of lactone-containing drugs like statins.

Statins are a main class of lipid lowering drugs, which inhibit the enzyme 3-hydroxy 3-methylglutaryl-coenzyme A (HMG-CoA) reductase and therefore block cholesterol synthesis in liver. One of HMG-CoA reductase inhibitors, atorvastatin is a highly effective drug with minimal side effects in the treatment of hypercholesterolemia in humans¹⁴. It may exert beneficial effects on atherosclerosis, independent of its cholesterol lowering ability, such as antioxidant properties and abilities to alter PON1 activity and gene expression^{12,15}. Although, statins strongly inhibit cholesterol synthesis *in vitro* and *in vivo* rat models, a hypocholesterolemic effect was not observed¹⁶. Thereby, the rat is a good model to examine other effects of statins independent from their most important action, cholesterol-lowering.

The objective of the current study was to investigate effects of atherogenic diet and atorvastatin therapy on PONs gene expression and distribution in rats tissues using real time polymerase chain reaction (PCR).

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Materials and Methods

Experimental Animals

The study was performed on young male Wistar rats. All animals were randomized into 3 groups. The animals were housed in metal cages (6 rats per cage) in an environmentally control room at the temperature of $20 \pm 2^\circ\text{C}$ and had free access to food and water during the experiment. Each group of 6 rats received 24 g of food in form of protein pellets per day. First group was on normal diet (ND) regime for eleven weeks, the second and the third group were on ND for one week and ten weeks on atherogenic diet (AD) which consisted of equally mixed and distributed 2% of cholesterol, 1% of Na-cholelate and 3% of *Helianthus annuus* oil on protein pellets. For the last six weeks, 1.14 mg atorvastatin per kg therapy was orally administrated by gavage to rats of the third experimental group (ADA). After 11th week, animals were euthanized with CO₂ asphyxiation. Blood was immediately collected from heart into EDTA-containing tubes. Whole blood was centrifuged for 10 min at 3000 rpm; plasma was separated, frozen and stored at -80°C until analysis. Portions of left liver lobe, epididymal white adipose tissue (WAT) and aorta were excised and immediately frozen in liquid nitrogen and kept at -80°C until ribonucleic acid (RNA) isolation was performed.

Methods

In plasma samples total cholesterol (TC), triglycerides (TG) and HDL-C were assayed by routine enzymatic methods using an ILab 300 + analyzer (Instrumentation Laboratory, Milan, Italy) and Randox Laboratories (Armdore, UK) reagents. LDL-C was calculated by Friedwald equation¹⁷. To determine the oxidative stress (OS) index in plasma, we used the thiobarbituric acid-reacting substances (TBARS) assay that measures the quantity of the malondialdehyde (MDA)-thiobarbituric acid 1 : 2 adduct described previously by Girotti *et al*¹⁸. The rate of nitroblue tetrazolium reduction was used to measure the level of superoxide anion radical (O₂⁻), as previously described¹⁹. Plasma superoxide dismutase (SOD) activity was measured according to the previously published method by Misra and Fridovich²⁰. Rate of PON1 activity towards diazoxon was measured spectrophotometrically using a UV/VIS Ultrospec III spectrophotometer (Pharmacia LKB, Cambridge, UK) in plasma according to the method described by Richter & Furlong²¹.

Prior to RNA isolation organs were homogenized in liquid nitrogen and TRIzolTM reagent (Invitrogen

Life Technologies, Carlsbad, CA, USA) was immediately added. RNA isolation was performed using slightly modified manufacturers protocol²². The concentration of isolated RNA was determined by spectrophotometric analysis of the absorbance at 260 nm. The ratios at 260 nm and 280 nm (A260/280) as well as at 260 nm and 230 nm (A260/230) were used to assess the protein and organic purity of the RNA solution. The integrity of the RNA samples and absence of genomic DNA contamination were demonstrated by native 1% agarose gel electrophoresis. All extracted RNAs were then stored at -80°C prior to reverse transcription (RT) reactions. RT and real-time PCR experiments were carried out on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan[®] reagent-based chemistry. RT was performed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. RNA was added in 2X RT master mix (containing MultiScribeTM reverse transcriptase, random primers and RNase inhibitor) and incubated in three steps thermal protocol: at 25°C for 10 minutes, at 37°C for 120 minutes and at 85°C for 5 seconds. RT negative controls (RNA in 2X RT master mix without MultiScribeTM reverse transcriptase) were also performed. Synthesised cDNAs were kept at -80°C until real time PCR analysis. Quantitative real-time PCR was performed using TaqMan[®] 5'-nuclease gene expression assays (Applied Biosystems, Foster City, CA, USA) for PON1 (Rn 01455909_m1), PON2 (Rn 01456019_m1) and PON3 (Rn 01500926_m1) genes. For evaluation of the potential reference gene, 9 genes were examined (GADPH, ACTB, TBP, Ywhas, B2M, HPRT1, UCB, HMBS and PPIA). The geNorm VBA applet for Microsoft Excel was used to determine the most stable gene from the set of tested genes. Hydroxymethylbilane synthase (HMBS) (Rn 00565886_m1) was proved to be the most stable housekeeping gene for all three tissues. Non-template controls (samples without cDNA) and RT negative controls were included in every experimental plate for each assay. Amplification cycling was carried out in a thermal cycler under the following conditions: AmpErase[®] UNG activation for 2 minutes at 50°C , then activation of AmpliTaq Gold[®] DNA polymerase for 10 minutes at 95°C and then 40 cycles of denaturation for 15 seconds at 95°C followed by annealing/extension for 1 minute at 60°C . The messenger RNA (mRNA) levels for each sample were

expressed relative to the HMBS mRNA levels to normalize the results of the genes of interest. The study protocol was reviewed and approved by the Ethical Committee for work with experimental animals University of Belgrade- Faculty of Belgrade.

Statistical Analysis

Comparisons of variables were performed using the Kruskal-Wallis test with Mann-Whitney *U* test for subgroup differences. The characteristics of study populations are presented as geometric mean and 95th confidence interval. Statistical analyses were performed using PASW® Statistic version 18 (Chicago, Illinois, USA) and Microsoft® Office Excel 2007. The value of *p* less than 0.05 was considered statistically significant.

Results

Rats weight in all three groups was measured at the beginning $m_{ND} = 178.0$ (169.25 - 185.25) g, $m_{AD} = 164.0$ (156.00 - 181.50) g, $m_{ADA} = 171.5$ (166.00 - 179.75) g and at the end $m_{ND} = 445.5$ (417.25 - 487.25) g; $m_{AD} = 493.0$ (480.50 - 508.00) g, $m_{ADA} = 491.5$ (485.25 - 504.50) g of the experiment. At the beginning there was no difference in rats body weight between the three groups. However, rats body weight was higher in AD than in ND group ($p = 0.045$). Also, rats body weight was higher in

ADA than in ND group ($p = 0.025$). There was no significant difference in rats body weight between AD and ADA groups.

Plasma lipid status parameters of tested animals are presented in Table 1. TC, LDL-C and TG levels were significantly higher in AD group ($p = 0.006$, $p = 0.004$ and $p = 0.004$, respectively) and ADA group ($p = 0.004$, $p = 0.002$ and $p = 0.002$, respectively) than in ND group of rats. We did not find significant difference in HDL-C levels between any of these three groups.

PON1 activity was significantly different between these three groups of rats ($p = 0.007$). The lowest PON1 activity was in ADA group compared to ND and AD groups ($p = 0.002$ and $p = 0.033$, respectively). TBARS concentration, O_2^- levels and SOD activity did not change significantly in rats plasma under the different treatments (Table 2).

In all three tissues and different treatment PON1, PON2 and PON3 mRNAs were detected. Effects of atherogenic diet on PONs expressions in rats liver, WAT and aorta were presented in Figure 1. Atherogenic diet had no influence on PON1 and PON3 mRNA levels, but PON2 mRNA levels in AD group were significantly higher ($p = 0.045$) than in ND group in liver [1.076 (1.0435 - 1.1328) and 0.958 (0.8631 - 1.0523), respectively] (Fig. 1A). PON2 and PON3 were significantly higher than PON1 in both,

Table 1 — Plasma lipid status parameters in tested animals on each diet regime and therapy

	ND	AD	ADA	<i>p</i>
TC, mmol/L	1.50 (1.32-1.60)	3.20 (2.45-3.35)*	2.95 (2.40-5.27)*	0.004
HDL-C, mmol/L	0.49 (0.41-0.53)	0.42 (0.38-0.52)	0.43 (0.37-0.50)	0.596
LDL-C, mmol/L	0.66 (0.53-0.80)	2.31 (1.77-2.78)*	2.34 (1.87-4.68)*	0.004
TG, mmol/L	1.01 (0.91-1.06)	2.57 (2.03-2.97)*	2.53 (2.02-4.86)*	0.004

Variables are presented as median (interquartile range)

p – for Kruskal Wallis test

* Significantly different from ND by Mann Whitney test ($p < 0.01$)

Table 2 — Oxidative stress and antioxidative defence markers in rats' blood

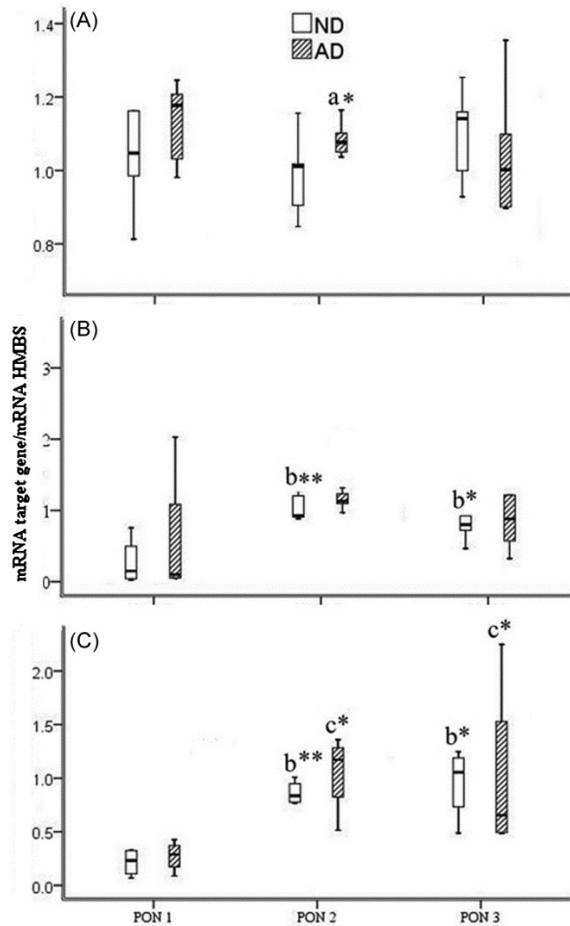
	ND	AD	ADA	<i>p</i> ¹
PON1, U/L	3393 (2903-3930)	2908 (2465-3073)	2049 ^{a*} , ^{b†} (1793-2369)	0.007
TBARS, μ mol/L	0.48 (0.38-0.57)	0.51 (0.43-1.27)	0.61 (0.57-0.68)	0.087
O_2^- Conc., μ mol NBT/min/L	41.50 (13.00-59.00)	28.50 (15.50-38.50)	14.50 (13.00-18.00)	0.235
SOD, U/L	112 (110-116)	110 (105-116)	118 (113-120)	0.257

¹ Variables are presented as median and interquartile range and compared by Kruskal Wallis test

a- significantly different from ND by Mann Whitney test

b- significantly different from AD by Mann Whitney test

* $p < 0.01$, † $p < 0.05$



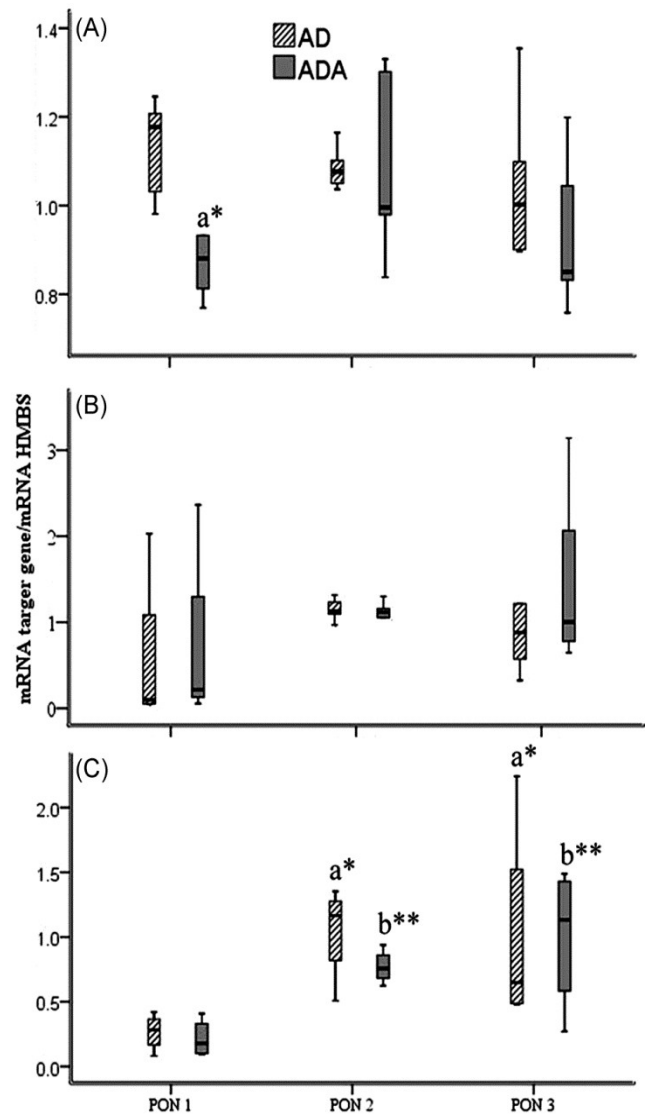
a - significantly different from PON2 expression in ND group
 b - significantly different from PON 1 expression in ND group
 c - significantly different from PON 1 expression in AD group
 * p<0.05, ** p<0.01

Fig. 1 — Differences between PON1, PON2 and PON3 mRNA levels in liver (A), WAT (B) and aorta (C) on normal diet (ND) and atherogenic diet (AD).

WAT ($p = 0.004$ and $p = 0.016$, respectively) and aorta ($p = 0.01$ and $p = 0.025$, respectively) in ND group of rats. Differences in mRNA levels between genes were kept only in rats aorta on AD but lost in WAT (Figs 1B & 1C).

Effects of atorvastatin treatment on PONs expressions in rats liver, WAT and aorta were presented in Figure 2. PON1 mRNA levels were significantly lower ($p = 0.028$) in ADA compared to AD group in liver [0.882 (0.8015 - 0.9955) and 1.177 (1.0063 - 1.2266), respectively] (Fig. 2A). PON2 and PON3 were significantly higher than PON1 in aorta ($p = 0.021$ for both genes) of AD and ADA groups ($p = 0.004$ and $p = 0.01$, respectively) (Figs. 2B & 2C).

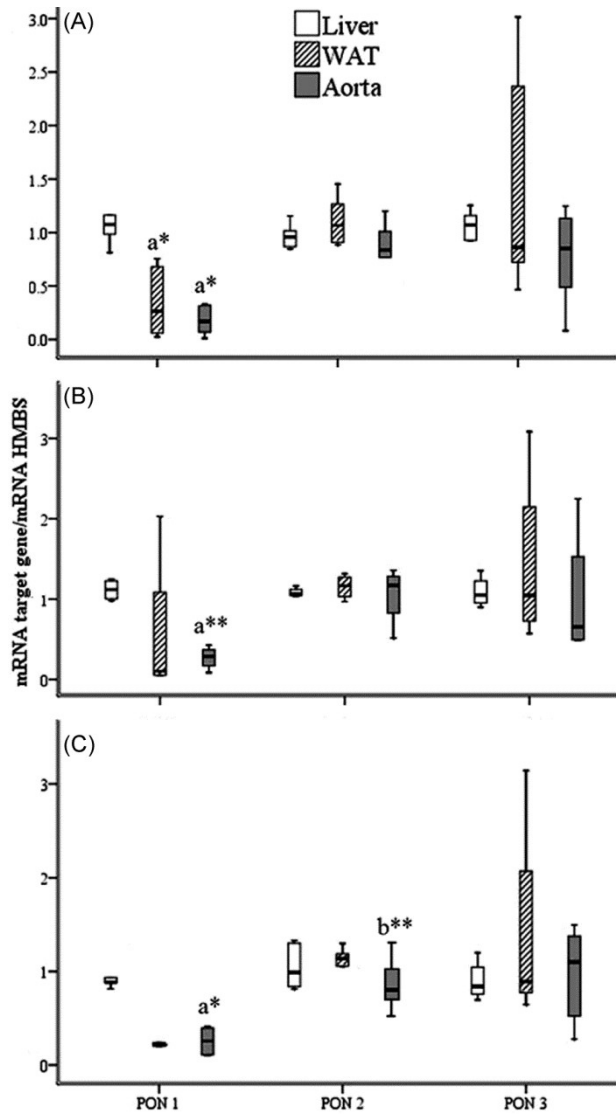
In Figure 3 we compared PONs' gene expression levels between tested organs within each treated



a - significantly different from PON1 expression in AD group
 b - significantly different from PON 1 expression in ADA group
 * p<0.05, ** p<0.01

Fig. 2 — Differences between PON1, PON2 and PON3 mRNA levels in liver (A), WAT (B) and aorta (C) on atherogenic diet (AD) and atherogenic diet with atorvastatin therapy (ADA).

group. In ND group we observed that PON1 mRNA levels were higher in liver than in aorta ($p = 0.004$) and than in WAT ($p = 0.004$), but there were no differences in mRNA levels of PON2 and PON3 between these organs (Fig. 3A). In the AD group, significant difference between PON1 mRNA levels in liver and aorta remained ($p = 0.014$), but significance was lost for liver and WAT. Still there were no differences in mRNA levels of PON2 and PON3 between these organs (Fig. 3B). In the ADA group,



a- significantly different from PON 1 expression in liver
 b- significantly different from PON 2 expression in WAT
 * p<0.01, ** p<0.05

Fig. 3 — PONs gene expression distributions between organs on ND (A), AD (B) and ADA (C) groups.

PON1 mRNA levels were still higher in liver than in aorta ($p = 0.004$). PON2 mRNA levels were higher in WAT than in aorta ($p = 0.04$). There were no differences in mRNA levels of PON3 between these organs (Fig. 3C).

Discussion

Members of PON family seem to have important part in keeping low oxidative state in blood and cells and therefore to prevent from atherosclerosis^{1,7}. Beside their anti-inflammatory and anti-atherogenic

properties, they were assumed to have a greater and more important role in cell functioning²³. Therefore, many studies were carried out in order to identify nutritional, physiological and pharmacological factors which might modify PONs expressions and activities^{10-12,24-27}. Atorvastatin is a drug of choice for reducing lipids in hypercholesterolemic patients. It was demonstrated that atorvastatin induces a significant reduction in TC, LDL-C, and TG levels, but the effect on HDL-C remains unchanged¹⁴. Despite its success in clinical practice, previously published study suggested that atorvastatin and all other inhibitors of HMG-CoA reductase were completely inactive in cholesterol-fed rats¹⁶. This was coherent with our study. Atorvastatin had no effects on TC, HDL-C, LDL-C and TG concentrations in hyperlipidemic rats (Table 1). In order to determine PONs gene expressions and activities most researches were conducted in normolipidemic mice or rats, because it is known that hyperlipidemia itself could, directly or indirectly through enhancing OS, modify PONs' gene expressions and activities^{1,28}. However, our results were not in agreement with this. Our study revealed that PON1 mRNA levels were not significantly different between ND and AD groups of rats either in liver, WAT or aorta (Fig. 1). However, treatment hyperlipidemic rats with atorvastatin showed that mRNA PON1 were significantly lower than mRNA levels in AD group (Fig. 2A). This is consistent with Gouedard's team¹² who published results where statins reduce PON1 activity and gene expression in cultured human hepatoma cell line (HuH7) decreasing PON1 promoter activity. Their results also suggested that the regulation of the PON1 gene itself determines PON1 activity level. In the study by Shih and colleagues²⁹, cholesterol-rich food lowered PON1 activity in serum and its gene expression in liver of mice (C57BL/6J) after 12 weeks treatment. Similar atherogenic diet used in our study did not change PON1 activity, but such atherogenic diet with atorvastatin therapy influenced PON1 activity to fall when compared to ND and AD groups (Table 2). Two studies by Beltowski^{15,30} were of interest as rares that showed fall in PON1 activity due to statin therapy. The results of his group indicated that cerivastatin and fluvastatin, but not pravastatin, reduces plasma and liver PON1 activity in normolipidemic rats in the short period of time (21 days). Obviously, long-term application of atorvastatin (42 days in our experiment) caused

adaptive downregulation of PON1 due to decreased requirement¹⁵. Moreover, it is possible that treatment with atorvastatin, which is *per se* antioxidant, might provide an indirect mechanism by which PON1 activity could fall off and its gene expression to be downregulated. OS/antioxidative defence (AOD) system disbalans was not evident after 10 weeks of atherogenic diet. Six weeks' atorvastatin therapy did not change TBARS, O₂⁻ concentrations and SOD activity (Table 2).

Chronic exposure of mice to high-cholesterol diet was shown to cause an increase in hepatic PON2 mRNA levels³¹. This observation is in line with ours which showed that PON2 expression was similarly up regulated by atherogenic diet (Fig. 1A). Moreover, increased PON2 expression has also been observed in response to atorvastatin which can upregulate PON2 expression in mouse macrophages²⁶. We lack to demonstrate the latter either in rats liver, WAT or aorta (Fig. 2). Unlike PON1 and PON2, PON3 expression was not affected either by atherogenic diet or atorvastatin therapy in studied organs. This was similarly to previously published data by Reddy's group, where PON3 mRNA was not altered in the livers of C57BL/6 mice fed by a high-fat diet². All together with our results, we may speculate that stable and relatively high PON3 expression in selected tissues may provide wider and distinct role than other two PONs in the prevention of atherosclerosis.

PON2 and PON3 exerted statistical higher expression than PON1 ($p < 0.01$ for both) in rats' WAT and aorta on ND (Figs. 1B & 1C). Under influence of AD, PON2 and PON3 expression levels remained higher than PON1 only in aorta (Fig. 1C). Similar were found for atorvastatin influence on PON2 and PON3 expression in aorta (Fig. 2C). They could play local anti-atherogenic and anti-oxidative effect as a defensive mechanism in the artery wall. We found that either AD or ADA did not alter PON1 and PON3 distribution between the organs (Fig. 3). Our results confirmed that PON1 was mainly expressed in liver compared to other organs²³, which was not changed by AD or ADA. PON3 had similar expression in all rat organs in all 3 groups. But in rats on ADA, PON2 expression levels were altered, becoming higher in WAT than in aorta ($p < 0.05$) (Fig. 3C), suggesting its potential role in lipid metabolism probably similar like PON1⁵.

To conclude, our results demonstrated that atherogenic diet induced up regulation of PON2 gene

and atorvastatin therapy induced down-regulation of PON1 gene in liver of hyperlipidemic rats. Atherogenic diet with atorvastatin therapy caused fall in PON1 plasma activity, probably due to the decreased requirement. Atherogenic diet and atorvastatin therapy did not change PON1 mRNA levels distribution between liver and aorta where they were always higher in liver than aorta. All three PONs have similar expression in liver, but PON2 and PON3 mRNA levels were higher than PON1 in aorta either on atherogenic diet or atorvastatin therapy, suggesting their important antiatherogenic role in aorta wall.

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