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Pig PON1: Expression and promoter methylation

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ABSTRACT

Atherosclerosis is an inflammatory disease promoted by oxidized low density lipoprotein (LDL). High density lipoprotein (HDL) is an important antioxidant, protecting LDL and itself from oxidation and by detoxifying the hydroperoxides from oxidized LDL. Paraoxonase, encoded by the PON1 gene, is an enzyme involved in oxidant defense by hydrolyzing oxidized lipids, including oxLDL, and in detoxification of organophosphate pesticides. Aging is the major risk factor for developing atherosclerosis and as paraoxonase is responsible for the antioxidant effect of HDL, aging might be accompanied by alterations in paraoxonase activity and/or expression.

Here we report the analysis of porcine *PON1* mRNA expression and *PON1* promoter methylation status during pig aging. *PON1* mRNA expression was detected exclusively in liver and kidney using RNAseq analysis. An increase in *PON1* transcript level was observed in porcine liver during aging. In contrast, no change in *PON1* mRNA expression was found in kidney. The DNA methylation status was examined in a discrete region of the *PON1* promoter and no significant changes were observed in liver during pig aging. However, a high methylation level was observed in occipital cortex correlating with no PON1 expression. Therefore, methylation might be one determinant of *PON1* expression.

1. Introduction

Atherosclerosis is an inflammatory disorder, a specific form of arteriosclerosis and is affecting the inner cell walls of arteries (Hansson et al., 2006; Libby, 2012). Atherosclerosis is characterized by endothelial dysfunction, thickening of artery cell wall, increased permeability of endothelium to lipoprotein, proliferation of intimal smooth muscle cell and necrosis (Libby, 1995). Predispository factors include age, sex, diabetes, obesity of hypertension. The aging process is considered to be the major risk factor for developing atherosclerosis (Smith, 1998; Lusis, 2000). The pathogenesis of atherosclerosis is promoted by low-density lipoproteins (LDL), which are particularly associated with initiation and progression of the disease mediated through in inflammatory process (Levitan et al., 2010). Oxidative stress is a dominant factor in the development of atherosclerosis (Katakami et al., 2009) and particularly, the oxidation of low-density lipoproteins (LDL) is involved in initiation and progression of atherosclerosis (Reaven et al., 1999). High density lipoprotein (HDL) is an important antioxidant protecting LDL and itself from oxidation and by detoxifying the hydroperoxides from oxidized LDL. Paraoxonase, encoded by the *PON1* gene, is an enzyme involved in oxidant defense by hydrolyzing oxidized lipids and in detoxification of organophosphate pesticides. PON1 catalyzes the hydrolysis of thiolactones and some xenobuitoics such as organophosphate esters, unsaturated aliphatic esters aromatic carboxylic esters and carbamates (La Du, 1992; Davies et al., 1996; Costa et al., 2005). Paraoxonase is also one of the enzymes involved in the toxifying hydroperoxides generated form oxidized LDL (Jansen et al., 2009). The atherosclerosis related cardiovascular diseases are increasing with aging and therefore an alteration in PON1 expression and/or activity might be a concomitant effect during aging. In humans, paraoxonase concentrations do not change during aging (Seres et al., 2004). However, the paraoxonase activity significantly declines with aging (Harman, 1992). PON1 activity in humans and rodents is low at birth and increases in older ages and into adulthood (Mueller et al., 1983; Cole et al., 2003). Mehdi and Rizvi (2013) demonstrated that PON1 arylesterase activity and plasma total thiols levels showed significant correlation with increasing levels of plasma protein hydroperoxides during aging. Mehdi and Rizvi (2013) also reported a decrease in plasma arylesterase activity of PON1 as a function of human age. Knock-down of *PON1* in human dermal microvascular cells resulted in cellular senescence and this suggests that PON1 may function as an aging-related protein (Lee et al., 2012). PON1 exerts its anti-atherogenic property by preventing/inhibiting accumulation of lipoperoxides and inhibition of lipid oxidation in LDLs (Tavori et al., 2009).

Abbreviations: ALB, albumin; APOA1, apolipoproteinA-1; CLU, clusterin; HDL, High density lipoprotein; LDL, Low density lipoprotein; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR

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Several polymorphisms have been identified in the PON1 gene, both in the coding region and in promoter region among those a Leu/Met in position 55 and a Arg/Gln in position 192 (Eckerson et al., 1983; Adkins et al., 1993). Paraoxonases, including PON1, is believed to play important roles in the innate immune system, due to their antoxidant properties and their ability to degrade N-acyl homoserine lactones (reviewed by Camps et al., 2017). PON1 is very likely to play a role in the pathophysiology of infection and associated inflammatory reaction. Therefore it is also possible that PON1 is involved in the neuroinflammation seen in Parkinson's disease and Alzheimer's disease.

Here we describe the spatial and temporal *PON1* expression and its possible function during pig aging. Also, we present the methylation status of a partial promoter sequence of *PON1*.

2. Materials and methods

2.1. Ethics statement

Pigs were housed and used in compliance with European Community animal care guidelines. Beforehand, the experimental procedures were submitted to the National Ethical Committee in Denmark. The pigs were sacrificed by an intravenous injection with 30 mg/kg Pentobarbital (Vipidan, Denmark).

2.2. Biological subjects

The following pigs were included in the methylation analysis and expression analysis: Danish Landrace pigs (id. 147, 3713 and 9071), one 7 year old female Danish Landrace, one 10 year old female Danish Landrace pig and one 12 year old female Danish Landrace pig.

2.3. Extraction of nucleic acids and cDNA synthesis

RNA was isolated from pig organs and tissues as previously described (Larsen et al., 2014). DNA was isolated from biological samples according to standard purification protocols (Green and Sambrook, 2012). Synthesis of the cDNA used for cloning was performed as previously described (Henriksen et al., 2009). cDNAs used for expression analysis were synthesized from RNA isolated from various adult porcine liver, kidney and occipital cortex using random hexamer primers (Roche) and the manufacturer's protocol.

2.4. Expression analysis

Total RNA was extracted from 10 different tissues from two boars using standard procedures, and RNA integrity was assessed by manual inspection of each RNA sample on a 2% agarose gel. The tissues employed were: cerebellum, frontal cortex, occipital cortex, hypothalamus, lung, spleen, liver, heart, kidney, and musculus longissimus dorsii. For library preparation, sequencing and estimation of FPKM values, the FPKM values for *PON1* presented in this study were drawn from a large dataset generated using next-generation sequencing. Briefly, mRNA sample preparation was accomplished using the mRNA-seq sample prep kit from Illumina and according to the manufacturer's protocol where 10 µg of each total RNA sample was employed in polyA mRNA selection using magnetic beads, followed by thermal fragmentation. Subsequently, the fragmented mRNA was reverse transcribed using reverse transcriptase, SuperScript II, and random primers. The cDNA was size-selected on a low-melting 2% agarose gel and fragments corresponding to sizes of 200 nucleotides were excised from the gel and DNA was recovered employing QIAquick gel extraction kit (QIAGEN). In order to enrich the libraries, 15 cycles of PCR were employed followed by purification by the QIAquick PCR purification kit (QIAGEN). The purified libraries were diluted to a concentration of 10 nM, and each library was sequenced as a multiplex of 10 samples per lane using 55 bp sequencing at a concentration of 20 pM on a Genome Analyzer (Illumina). Subsequently, all fragments were mapped to build 10.2 including mitochondrial DNA of the *Sus scrofa* genome applying TopHat version 1.3.3. (Trapnell et al., 2009) and the aligned reads were processed and assembled into transcripts by Cufflinks version 0.8.0. (Trapnell et al., 2010). Furthermore, Cufflinks estimates the relative abundance of each transcript and reports it in fragments per kilobase of exon per million fragments mapped (FPKM), and hence the FPKM values for *HTR2C* for each animal for all tissues were estimated.

Table 1
List of oligonucleotide primers and probes used in cloning, sequencing, expression and methylation analyses of the porcine *PON1* gene.

Primer	Sequence (5′ - 3′)
PON1-PSF2	TTATTTAGTTTAGGTAGTTCG
PON1-PSR	[Btn]CAAAATTAATCCCACCCCTC
PON1-S1	TTTTTTTTAAAGTGTTTTAG
PON1-S2	GATTTTAAGTTTGCGGTG
PON1-RTF	CGAAGCTGATGGTGCTCA
PON1-RTR	GAAGACCGATGATCCCTGAA
PON1 probe	GGGCTGGG
GAPDH-RTS	CATGGCCTTCCGTGTTCCTA
GAPDH-RTAS	CCCTCAGATGCCTGCTTCA
GAPDH probe	VIC-CATCACTGCCACCCAGA

2.5. Methylation analysis of PON1

The methylation status of *PON1* promoter was determined by bisulfite sequencing of individual clones from PCR and by pyrosequencing. Genomic DNA from pig liver, kidney and occipital cortex was isolated and bisulfate-treated using the EZ DNA methylation kit (Zymo Research) following the manufacturer's instructions. One primer-set was used in pyrosequencing of bisulfite-treated DNA (Table 1). PCR amplification primers and sequencing primer were designed using the PyroMark Assay Design software (Qiagen). First-round PCRs were using bisulfate-treated DNA with forward and biotinylated reverse primers (Table 1) and using the Qiagen PyroMark PCR kit. Twelve microliters of PCR product were used for pyrosequencing applying the PyroMark Q24 system from Qiagen.

3. Results and discussion

3.1. Analysis of the porcine PON1 amino acid sequence

The porcine *PON1* gene, previously cloned by Xie et al. (2010), encodes a protein of 355 amino acids. The PON1 protein contains a 15 amino acid signal peptide which upon cleavage results in a mature protein of 340 amino acid residues. We performed a multiple alignment of PON1 protein sequences from human, pig, cow and a deduced partial PON1 amino acid sequence from bowhead. The alignment, shown in Fig. 1, demonstrated a high homology of the porcine PON1 amino acid sequence with that of human (86%), bovine and bowhead (82%). Within the 96 amino acids of the PON1 proteins aligned eleven substitutions are found in the porcine compared with the human sequence. Interestingly, the Q192R polymorphism previously characterized as a risk factor for coronary artery disease is not conserved in the four compared PON1 protein sequences. In the porcine PON1 sequence a threonine residue is found at position 192 and in cow and bowhead an arginine is situated at this particular positon. An arginine residue is also found in PON1 sequences from mouse, rat and chimpanzee (data not shown). The substitution of a glutamine (Q) residue with an arginine, which is present in most other PON1 proteins at position 192 raise an interesting question. Is Q192R polymorphism a causal risk factor for development of coronary artery disease or is affecting interaction with other proteins? Studies of the association of the *PON1* Q192R polymorphism with coronary artery disease are inconsistent and conflicting. Several studies have reported a positive correlation of Q192R with