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EDITORIAL

Measurement of serum paraoxonase-1 activity in the evaluation of liver function

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Abstract

Paraoxonase-1 (PON1) is an esterase and lactonase synthesized by the liver and found in the circulation associated with high-density lipoproteins. The physiological function of PON1 seems to be to degrade specific oxidized cholesteryl esters and oxidized phospholipids in lipoproteins and cell membranes. PON1 is, therefore, an antioxidant enzyme. Alterations in circulating PON1 levels have been reported in a variety of diseases involving oxidative stress including chronic liver diseases. Measurement of serum PON1 activity has been proposed as a potential test for the evaluation of liver function. However, this measurement is still restricted to research and has not been extensively applied in routine clinical chemistry laboratories. The reason for this restriction is due to the problem that the substrate commonly used for PON1 measurement, paraoxon, is toxic and unstable. The recent development of new assays with non-toxic substrates makes this proposal closer to a practical development. The present editorial summarizes PON1 biochemistry and function, its involvement with chronic liver impairment, and some aspects related to the measurement of PON1 activity in circulation.

Key words: Lipoproteins; Liver cirrhosis; Liver function tests; Oxidative stress; Paraoxonase-1

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INTRODUCTION

The paraoxonase (PON) enzyme family comprises 3 members, PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7q21-22[1]. In humans, PON1 and PON3 are mainly found in the circulation bound to high-density lipoproteins (HDL)[2]. Conversely, PON2 is an intracellular enzyme[3]. Their physiological roles have not been completely ascertained. PON1 has esterase and lactonase activities[4] and is involved in protection against xenobiotic toxicity[5]. PON2 and PON3 have only lactonase activity[6]. All the PONs are able to reduce low density lipoprotein (LDL) oxidation[7], while PON2 reduces cellular oxidative stress and prevents apoptosis in vascular endothelial cells[8]. PON1 is the best known among these enzymes. Alterations in circulating PON1 levels have been reported in a variety of diseases involving oxidative stress. These include cardiovascular disease, Alzheimer's disease, chronic renal failure, HIV-infection, metabolic syndrome, and chronic liver impairment[9]. As such, increased knowledge of the physiological significance of PON1 and its involvement in human pathology would be of critical importance in the years to come. In the present article we review fundamental concepts regarding PON1 biochemistry and function, and the relationships with chronic liver diseases. We also discuss the possible application of its measurement in serum for an improved evaluation of hepatic function.

PON1 IS AN ANTIOXIDANT ENZYME

The first approximation to the physiological role of PON1 was suggested by Mackness *et al*[10]. The authors investigated the protection against copper-induced LDL oxidation *in vitro* provided by purified PON1. They observed that this enzyme prevents the generation of

lipoperoxides during the process of LDL oxidation. Further studies from this and other groups reached the conclusion that PON1 protects LDL and HDL from lipid peroxidation by degrading specific oxidized cholesteryl esters and specific oxidized phospholipids contained in oxidized lipoproteins[11-13]. PON1 is, in turn, inactivated by oxidized lipids. This was shown by Aviram *et al*[14], who demonstrated that the incubation of PON1 *in vitro* with oxidized palmitoyl arachidonoyl phosphatidylcholine, lysophosphatidylcholine, and oxidized cholesteryl arachidonate, inactivated PON1 activity, as well as did oxidized LDL. Cysteine-284 was required for this effect of oxidized lipids on PON1 because, in recombinant PON1 in which this aminoacid had an induced mutation, no inactivation was observed. A further article from the same group showed that, under oxidative stress, PON1 may be inactivated by *S*-glutathionylation, a redox regulatory mechanism characterized by the formation of a mixed disulfide between a protein thiol (i.e. cysteine-284) and oxidized glutathione[15].

Identifying the native function of PON1 has, for a long time, been hampered by confusion with respect to the structure and mechanism-of-action of this enzyme. Several studies established the primordial function of PON1 as that of a lipolactonase[16-18] which subsequently evolved new substrate specificities. These studies also established that the preferred substrates of PON1 are 5- and 6-membered ring lactones, typically with aliphatic side-chains[19]. A model has been proposed to link lactonase activity and the degradation of lipid peroxides[20] by which oxidized lipids containing hydroxyl groups at the 5'-position could be lactonized by PON1 to yield lysophosphatidylcholine and δ-valerolactone products. As such, according to this hypothesis, the PON1 ability to degrade lipid peroxides is secondary to its lipolactonase activity.

PON1 IS ESSENTIALLY SYNTHESIZED BY THE LIVER

PON1 is found mainly in serum and in the liver. Northern blot analysis performed in human and rabbit tissues detected *PON1* mRNA only in the liver, although reverse transcriptase PCR (RT-PCR) studies in mice identified *PON1* mRNA in liver, kidney, heart, brain, intestine, and lung[1]. It seems highly likely that the liver is the main source of serum PON1 since it is the organ with the highest *PON1* gene expression, and where an important percentage of HDL is synthesized and secreted into the circulation. Over the last 15 years, there have been several attempts to purify hepatic PON1 to homogeneity with the aim of comparing its properties with those of the serum enzyme. This task is complicated by the hepatic PON1 being an enzyme associated with membrane vesicles derived from the endoplasmic reticulum[21].

In 1993, the first method for the partial purification of rat liver PON1 was published[22]. Essentially, the process consisted of the preparation of microsomes, solubilization with Triton X-100, adsorption on to hydroxyapatite, and chromatography with DEAE-52 cellulose to yield a 77-fold purified product. Later, Huang *et al*[23] isolated PON1 from mouse hepatic microsomes, and Rodrigo *et al*[24] in 1997, purified rat liver PON1 to homogeneity. They achieved a 415-fold purified product by using hydroxyapatite adsorption followed by three chromatography steps including DEAE-Sepharose, affinity chromatography, and Mono Q HR fast-performance liquid chromatography. The N-terminal sequence and two internal sequences of the purified protein were similar to those of rabbit and human PON1 of serum and mouse liver PON1. Subsequent studies with rat and human liver PON1 demonstrated many biochemical characteristics in common with those of serum PON1. These included optimum pH, substrate affinity (K_M), kinetic constants, heat inactivation, and calcium requirement[25]; all of which strongly suggested a high degree of identity between both enzymes.

What is the true role of hepatic PON1? If HDL-bound serum PON1 is an antioxidant enzyme, it may not seem illogical to infer that a similar function could apply to intracellular PON1. Indeed, liver microsomes are the major sites for the catabolism of xenobiotic compounds, in the course of which process an increased production of free radical species is observed. Rodrigo *et al*[26] observed PON1 protein expression mainly in the hepatocytes from the centrolobular region, thus supporting the hypothesis of intrahepatic PON1 participation in oxidative by-product inactivation.

OXIDATIVE STRESS AND CHRONIC LIVER IMPAIRMENT

Increased oxidative stress and inflammation play a fundamental role in the onset and development of liver diseases. The most important causes of chronic liver disease are alcohol abuse, obesity, and hepatitis C virus infection.

Alcoholic liver disease (ALD) comprises a broad spectrum of hepatic alterations ranging from steatosis and minimal injury to advanced fibrosis and cirrhosis[27]. The involvement of oxidative injury in ethanol toxicity has emerged from reports showing that alcohol-fed animals and patients with ALD present with a high content of lipid peroxidation products in their livers and in the circulation[28]. Oxidative stress associated with ethanol intake comes mainly from reactive oxygen species (ROS) generated by the mitochondrial respiratory chain and cytochrome P4502E1 from hepatocytes, and the NADPH oxidase from Kupffer cells and recruited macrophages[29]. The impairment of mitochondrial lipid oxidation is one of the mechanisms responsible for hepatic fat accumulation[30]. Pan *et al*[31] reported that lipid peroxidation reduces hepatic lipoprotein secretion by enhancing the degradation of newly synthesized apolipoproteins and this effect, together with alterations in lipoprotein glycosylation in the Golgi apparatus[28], might contribute to microvesicular steatosis. Further evidence suggests that alcohol-induced oxidative stress interferes with the regulation of lipid synthesis by the peroxisome proliferator-activated receptor-α and the sterol

regulatory element binding protein 1[32]. The possible role of oxidative stress in promoting an inflammatory reaction in ALD has emerged from the observation that ethanol-induced lipid peroxidation increases the hepatic production of cytokines, growth factors, and collagen[33-35].

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are hepatic lesions which appear frequently in obese and diabetic individuals despite the fact that they may not have a history of alcohol abuse. These lesions resemble those of ALD, and are characterized by steatosis, hepatocyte hydropic degeneration, and inflammatory infiltrates. In addition, alterations in mitochondrial shape and function, and varying degrees of fibrosis are usually found[36]. NAFLD is an emerging lesion in modern societies, and will become more prevalent in the future, as it is associated with insulin resistance, metabolic syndrome, diabetes, and obesity. Oxidative stress plays a pivotal role in the evolution of "benign" steatosis to the more severe NASH. Several studies have shown that mitochondria in patients with NASH are abnormal from both the morphological and the functional points of view and, as in ALD, alterations in the fatty acid β-oxidation promote increased free radical production and lipid peroxidation[36]. The consequences of oxidative stress in NASH would be similar to those of ALD, with altered lipoprotein synthesis and secretion, an inflammatory reaction, and fibrosis.

Hepatitis C virus (HCV) is a major cause of viral hepatitis. In the USA, about 4 million people are infected, and 35 000 new HCV cases are estimated to occur every year[37]. The infection by this virus frequently does not resolve, and about 80% of the infected individuals become chronic carriers who may then progress to the most severe forms of liver impairment, as cirrhosis or hepatocellular carcinoma. Lipid peroxidation products, aldehydes as 4-hydroxynonenal, and 8-hydroxyguanosine (a marker of oxidative DNA damage) are elevated in HCV infection[38]. The increased oxidative stress may be explained by chronic inflammation and the generation of free radicals by Kupffer cells and recruited macrophages. The NS3 protein of HCV has been found to activate Nox 2 protein from macrophages, leading to increased generation of ROS that can exert oxidative stress to the nearby cells[39]. Furthermore, studies have indicated that HCV can directly induce oxidative stress in hepatocytes. HCV core gene expression has been associated with increased ROS, decreased reduced glutathione content, and increased thioredoxin in parenchymal cells. Recent studies showed that HCV core proteins bind to the outer mitochondrial membrane resulting in mitochondrial dysfunction by Ca^{2+} accumulation. These alterations would inhibit electron transport and promote ROS production[37]. Another HCV protein, NS5A, has also been reported to increase free radical production by Huh7 cells[40]. As in ALD and NASH, increased oxidative stress would produce a multifactorial reaction involving the synthesis of pro-inflammatory and pro-fibrogenetic cytokines and chemokines.

Therefore, it seems evident that chronic liver diseases share common biochemical alterations irrespective of their etiology. They are all accompanied by an increased oxidative stress secondary to mitochondrial abnormalities, promoting changes in lipid and lipoprotein metabolism, fat accumulation, an exacerbation of the inflammatory reaction due to increased cytokine synthesis, and extracellular matrix deposition.

THE MEASUREMENT OF SERUM PON1 ACTIVITY

There are no standardized methods for measuring PON1 esterase activity. The most widely used method is the hydrolysis of paraoxon. However, this method is not free of drawbacks, because paraoxon is very unstable and extremely toxic. The solution to the former problem is to prepare the reagent immediately before use. The solution to the latter problem requires that the stock solutions be handled in an air-extraction cupboard and the operator to take appropriate safety precautions such as wearing masks and gloves to protect against accidental contact or inhalation of the toxic fumes. Recent significant advances in the search for reliable PON1 lactonase activity assays may facilitate the measurement in a routine clinical chemistry laboratory setting. A new serum test based on this capacity of PON1, and employing 5-thiobutyl butyrolactone (TBBL) as a substrate, was recently proposed[41,42]. TBBL is a synthetic chromogenic lactone that resembles the natural lipolactone substrate of PON1. The method enables PON1 activity to be measured using a more 'physiological-like' substrate.

SERUM PON1 ACTIVITY IN CHRONIC LIVER IMPAIRMENT

In chronic liver diseases, oxidative stress influences the pathophysiological changes leading to liver cirrhosis and to hepatocellular carcinoma. Since PON1 exerts a protective effect against oxidative stress, it is logical to find an association between this enzyme and liver impairment. Ferre *et al*[43] observed, in rats with carbon tetrachloride-induced fibrosis, that an inhibition of hepatic PON1 activity was an early biochemical change related to increased lipid peroxidation and liver damage. They investigated the relationships between hepatic microsomal PON1 activity, lipid peroxidation and the progress of the disease in this experimental model. They found that PON1 activity decreased while lipid peroxidation increased in carbon tetrachloride-administered rats while the addition of zinc, which possesses antioxidant and anti-fibrogenetic properties, was associated with enhanced PON1 activity and a normalization of lipid peroxidation. This study suggested that PON1 activity may be involved in the defence against free radical production in liver organelles.

Pioneer studies in the 1970's observed for the first time a significant decrease in serum PON1 activity in small groups of patients with liver cirrhosis[44,45]. This results were confirmed by Ferre *et al*[46,47] in a wider series of patients with various degrees of chronic liver damage. These latter studies noted a significant decrease of serum

PON1 activity in patients with chronic hepatitis, and an even greater decrease in cirrhotic patients, compared to a control group. In alcoholic patients, the effects of alcohol intake on serum PON1 levels depend on the degree of liver dysfunction. In a study conducted in chronic alcohol abusers, subjects were classified into several sub-groups according to their degree of liver disease. The results demonstrated that serum PON1 activity was decreased in alcoholic patients, and that the magnitude of the alteration was related to the degree of liver damage[48]. These findings differ from those described in normal volunteers reporting moderate alcohol consumption, and in whom serum PON1 activity and HDL cholesterol were found to be slightly increased[49]. Changes in serum PON1 activity has also been studied in relation to outcomes of liver transplantation in patients with severe liver disease[50]. The serum PON1 activity was low, but tended to increase, in liver transplanted patients when the hepatic arteries had become blocked. Since PON1 activity is closely related to the recovery of liver function, its measurement could provide more accurate information on the success, or otherwise, of the liver transplant.

Serum PON1 measurement has been proposed as an useful test for the evaluation of the degree of liver impairment. Clinical diagnosis of chronic liver impairment and/or liver fibrosis is currently conducted *via* the invasive procedure of needle biopsy followed by histological evaluation. This procedure has important drawbacks, including a significant mortality rate (1/10 000-1/1000), sampling error, and subjectivity. Therefore, the development of non-invasive tests for the diagnosis of liver disease and the extent of the disease is an important goal of current research. Unfortunately, most of the individual laboratory tests to assess liver impairment have low specificity and sensitivity and, hence, the standard approach is to perform a battery of several tests followed by an algorithmic evaluation of the results. It is for this reason that several years ago, Ferre *et al*[46] proposed the addition of serum PON1 paraoxonase activity measurement as a biomarker of liver impairment. Serum PON1 measurement has an important feature in that the measured value is inversely related to the degree of liver derangement i.e. it decreases while most of the standard laboratory test values increase with the extent of the disease. Thus, PON1 measurement makes an additional contribution in improving current algorithms, and the ratios between tests. These authors estimated, by multiple logistic regression analysis, that the addition of paraoxonase measurement to a battery of standard liver function tests increased the overall sensitivity up to $\geqslant$ 90%, while keeping the specificity close to 100%. However, the measurement of this enzyme is, to-date, restricted to research laboratories and has not been extensively applied as yet in routine clinical chemistry laboratories, due to the problems associated with the use of paraoxon as a substrate. These drawbacks preclude full automation of PON1 measurement and, as such, can rarely be justified for inclusion in panels of standard biochemical tests. The recent development of new assays, such as the TBBL lactonase assay, makes this proposal closer to practical development. The TBBL assay has been shown to be equivalent to the paraoxon assay in terms of diagnostic accuracy[42], but with better safety of the TBBL substrate for the laboratory worker, and makes the lactonase measurement a strong candidate for inclusion into routine clinical laboratory testing of liver impairment, or for the study of other diseases involving oxidative stress.

CONCLUSION

Research into paraoxonases has flourished over the last 10 years. It seems now evident that PON1 is a lactonase with the ability to degrade lipid peroxides in lipoproteins and in cells, and that plays a protective role against oxidative stress and inflammation, which are key processes involved in the pathophysiology of chronic liver diseases. In the years to come, more reliable, practical, and accurate methods to measure PON activity and concentration will become available and these will facilitate more research in this field, and also enable the addition of PON measurement to the battery of routine analyses in clinical chemistry laboratories.

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