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Full Length Research Paper

In vitro inhibition of the paraoxonase from human serum with sulfonamide

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This study was conducted to determine the *in vitro* effects of sulfonamide on human serum paraoxonase (PON1) activity. The enzyme was purified by two-step using ammonium sulfate precipitation and sepharose-4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography. Sulfonamide was an effective inhibitor on purified human serum PON1 activity for phenylacetate and paraoxon as substrates with IC_{50} values of 0.22 and 0.81 mM, respectively. The kinetics of interaction of sulfonamide with the purified enzyme indicated a different inhibition pattern for two substrates. Sulfonamide showed a non-competitive inhibition with K*i* of 0.0037 ± 0.0009 mM for phenylacetate and competitive inhibition with K*i* of 0.0057 ± 0.0002 mM for paraoxon.

Key words: Paraoxonase, sulfonamide, inhibition, *in vitro*.

INTRODUCTION

Esterases have major roles in the hydrolysis of a number of prodrugs in human and experimental animals (Satoh, 1987; Obermeier, et al., 1996; Tang and Kalow, 1995; Senter et al., 1996; Krasny et al., 1995). They are classified in three groups (A, B and C) on the bases of their reactivity. Interindividual variation in the activity of the esterases is an important factor that influences both the pharma-cological and toxicological effects of prodrugs in humans (Williams, 1985). Large interindividual variations in esterase activity have been reported for carbonic anhydrase (EC 4.2.1.1) (Verpoorte et al., 1967), butylcholinesterase (EC 3.1.1.8) (McGuire et al., 1989), carboxylesterase (EC 3.1.1.1) (Hosokawa et al., 1995), paraoxonase/arylesterase (EC 3.1.8.1) (Playfer et al., 1976) and *S*-formylglutathione hydrolase (EC 3.1.2.12) (Eiberg and Mohr, 1986). One of the A-esterase of paraoxonase/arylesterase (PON1, EC 3.1.8.1) is a 355 aminoacid glycoprotein, which is sythesized in the liver and secreted into the blood, where it associated with HDL (high-density lipoprotein) (Hassett et al., 1991). It is a member of a three gene family consisting of PON1, PON2 and PON3 located on human chromosome 7 (Primo-Parmo et al., 1996). The PON is a hydrolase family with quite broad substrate specificity. PON1 was the first identified protein and thus the most studied. Early research focused on the observation that PON1 could hydrolyze organophosphorus (OP) compounds, including paraoxon (from which it takes its name), the insecticides parathion and chlorpyriphos as well as the nerve agents sarin and soman (Davies et al., 1996). It also hydrolyses aliphatic lactones such as dihydrocoumarin, γ-butyrolactone and homocysteine thiolactone (Billecke et al., 2000; Jakubowski, 2000). Its lactonase activity on lovastatin, simvastatin and spirinolactone also has been reported (Billecke et al., 2000). However, primary physiological role of PON1 is to protect low-density lipoproteins (LDL) from oxidative modifications (Durrington et al., 2001). Oxidized LDL is believed to play a central role in mono-cyte chemotaxis and macrophage differentiation, which are early events in the progression of arterosclerosis, whereas HDL destroys these biologically active oxidized lipids (Lusis, 2000). PON1, the major enzyme responsible for this protective effect, is associated with the HDL particle (Mackness et al., 1993). PON1 also protects phospholipids in HDL from oxidation (Aviram et al., 1998). More recently, PON1 has been shown to play a role in the metabolism of pharmaceutical drugs (Costa et al., 2003).

The sulfonamides constitute an important class of drugs, with several types of pharmacological agents possessing antibacterial (Drew, 2000), antitumor (Supuran, 2002), anticarbonic anhydrase (Supuran et al., 2003; Supuran et al., 2002; Supuran and Scozzafava, 2001), diuretic (Maren, 1976; Supuran et al., 1996), hypoglyce-

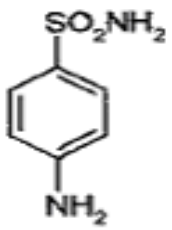

Figure 1. Structure of sulfanilamide

mic (Boyd, 1988) antithyroid (Thornber, 1979) or protease inhibitory activity (Ogden et al., 2001; Supuran and Scozzafava, 2002; Scozzafava and Supuran, 2000).

The very simple sulfanilamide (Figure 1) lead molecule afforded the development of all these types of pharmacological agents with such a wide variety of biological actions, as antibacterial agent sulfathiazole, the carbonic anhydrase inhibitor acetazolamide (clinically used for over 45 years), the widely used diuretic furosemide, the hypoglycemic agent glibenclamide the anticancer sulfonamide indisulam (in advanced clinical trials), the aspartic HIV protease inhibitor amprenavir used for the treatment of AIDS and HIV infection, or the metalloprotease inhibitors.

Considering the sulfonamides constitute an important class of drugs and also the importance of PON1 activity for atherosclerosis and antitoxicity, the *in vitro* inhibitory effects of sulfonamide on human serum PON1 was first time experimentally investigated for two substrates in this present study. Therefore, human serum PON1 was purified by our novel two step procedure using ammonium sulfate precipitation and sepharose-4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography (Sinan et al., 2006).

MATERIALS AND METHODS

Materials

Sepharose 4B, L-tyrosine, 1-napthylamine and protein assay reagents were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck.

Enzyme assay

PON1 activity was determined using paraoxon as a substrate and measured by increases in the absorbance at 412 nm due to the formation of 4-nitrophenol, by the method described by Gan et al. (1991). Briefly, the activity was measured at 37°C, by adding 50 µl of serum to 1 ml Tris/HCl buffer (100 mM, pH 8.0) containing 2 mM $CaCl_2$ and 5.5 mM of paraoxon. Enzymatic activity was calculated using the molar extinction coefficient 17 $100M^{-1}$ cm^{-1}. One unit of PON1 activity is defined as 1 µmol of 4-nitrophenol formed perminute under the above assay conditions.

Arylesterase activity was also measured spectrophotometrically. The assay was started by addition of the purified enzyme to the reaction mixture containing 1 mM phenylacetate in 20 mM Tris/HCl (pH 8.0) and 2 mM $CaCl_2$ and the increase in absorbence was recorded at 270 nm (Eckerson et al., 1983). Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. Enzyme activity was calculated using the molar extinction coefficient of 1310 M^{-1} cm^{-1}. One unit (U) is defined as 1 µmol phenylacetate hydrolysed per minute.

Total protein determination

The absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulfate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford (Bradford, 1976), with bovine serum albumin as a standard.

Purification of paraoxonase

Human serum was isolated from 50 ml fresh human blood and put into a dry tube. For this, the blood samples were centrifuged at 1500 rpm for 15 min and the serum was removed. Serum paraoxonase was precipitated by ammonium sulfate (60 - 80%) and it was collected by centrifugation at 15000 rpm for 20 min, redissolved in 100 mM Tris–HCl buffer (pH 8.0). The hydrophobic gel, including Sepharose 4B, L-tyrosine and 1-napthylamine, was synthesized for purification of the enzyme as described by Sinan et al. (2006). The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8.00) including 1 M ammonium sulfate and 15 mL enzyme solution was loaded. The paraoxonase was eluted with a linear of 1.0 - 0.0 M ammonium sulfate gradient in the 0.1 M Na_2HPO_4 buffer (pH 8.0). The purified PON1 enzyme was stored at 4°Cin the presence of 2 mM calcium chloride in order to maintain activity.

In vitro inhibition kinetic studies

For the inhibition studies, different concentrations of sulfonamide were added to the each enzyme activity. Paraoxonase and arylesterase activities with sulfonamide were assayed by following the hydration of paraoxon and phenylacetate, respectively. Activity (%) values of paraoxonase for eight different concentrations of sulfonamide were determined by regression analysis using Microsoft Office 2000 Excel. Paraoxonase activity without a sulfonamide was accepted as 100% activity. For the sulfonamide having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC_{50} values) was determined from the graphs. In addition, *Ki* values of sulfonamide against two substrates were determined for paraoxonase and arylesterase activities. In order to obtain K_M and V_{max} values of the enzyme for paraoxon at optimum pH (pH: 8.0) and temperature (37°C) the activity was measured at eight different substrate concentrations. K_M and V_{max} values were determined by means of Lineweaver–Burk graphs. The final concentration of sulfonamide 0.47 and 0.95 mM for paraoxon as substrate, 1.4 and 2.3 mM for phenylacetate as substrate was added to the mixture, reaction resulting in two different fixed concentrations of the sulfonamide. K_i values were calculated from Lineweaver–Burke graphs.

RESULTS

The human serum PON1 was purified sequentally by ammonium sulfate precipitation and Sepharose 4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography. The purity of the enzyme was confirmed with

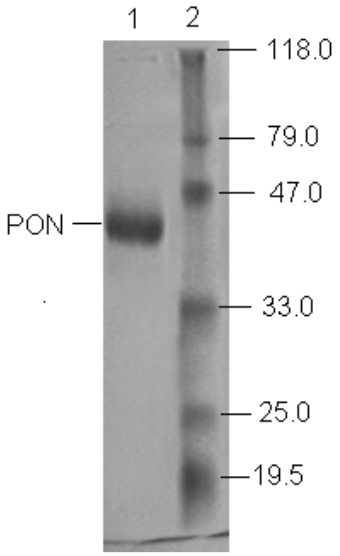

Figure 2. SDS/PAGE of human serum paraoxonase. The pooled fractions from ammonium sulfate precipitation and hydrophobic interaction chromatography (Sepharose-4B, L-tyrosine, 1-Napthylamine) were analysed by SDS/PAGE (12 and 3%) and revealed by Coomasie Blue staining. Experimental conditions were as described in the text. Lane 2 contained 3µg of various molecular-mass standarts: β-galactosidase, (118.0), bovine serum albumin (79.0), ovalbumin (47.0), carbonic anhydrase, (33), β-lactoglobulin (25.0), lysozyme (19.5). Purified human serum paraoxonase (lane 1) migrated with a mobility corresponding to an apparent M_r 45.0 kDa.

Table 1. The Effects of sulfonamide on purification by human serum PON1 and kinetic analysis of the inhibition.

Substrate	IC_{50} (mM)	K_i (mM)	Inhibition type
Paraoxon	0.81	5.7±0.2	Competitive
Phenylacetate	0.22	3.7±0.9	Noncompetitive

SDS gel electrophoresis (Figure 2).

Sulfonamide, constitutes an important class of drugs, was chosen for investigation of inhibition effects. The sulfonamide concentrations causing up to 50% inhibition was determined from the regression analysis graphs with paraoxon and phenylacetate as substrates and IC_{50} values were 0.81 and 0.22 mM, respectively (Table 1). Sulfonamide significantly inhibited the purified PON1 activity in a dose-dependent fashion (Figure 3 A, B). It exhibited a stronger inhibition for phenylacetate substrate than paraoxon on the enzyme activity. The interaction kinetics of sulfonamide against the paraoxon and phenylacetate were determined using 0.47 0.95 and 1.4 mM, 2.3 mM concentrations, respectively. The corresponding K_i values were calculated by the method of Lineweaver-Burk. Sulfonamide competitively inhibited the enzyme against paraoxon and exhibited a non-competitive inhibition against phenylacetate (Figure 3 C, D).

DISCUSSION

The mammalian PONs is divided into three subfamilies (Primo-Parmo et al., 1996). PON1 is by far the most investigated member of the family and became the subject of intensive research owing to its ability to inactivate various organophosphates, including nerve gases and pesticides, which present both an environmental risk and a terrorist threat. The name is derived from paraoxon, the matabolite of the common pesticide parathion, which is hydrolyzed by PON1 with modest catalytic efficiency ($k_{cat}/K_M \approx 10^4$ M^{-1}, s^{-1}). PON1 has been reported to be involved in drug metabolism and is used for drug inactivation (Biggadike et al., 2000). Research in the past decade has also shown that PON1 has antiatherosclerotic activity (Lusis, 2000). *In vitro* assays indicate that it inhibits lipid oxidation of the low-density lipoprotein and mediates the efflux of cholesterol from macrophages (Costa et al., 2003). PON1 has an appreciable aryl esterase activity, with phenyl acetate being a typical substrate ($k_{cat}/K_M \approx 10^6$ M^{-1}) (Khersonsky and Tawfik, 2005). It was reported that the Km values of paraoxon and phenylacetate as substrate 0.86 ± 0.02 mM and 1.3 ± 0.2 mM, respectively (Khersonsky and Tawfik, 2006).

The inhibitory effects of sulfonamide on purified human serum PON1 enzyme activity using paraoxon and phenylacetate as substrates were shown for the first time. The usage of two different substrates for inhibition studies enables researches to better understand the role and molecular structure of the enzyme. The inhibition studies of PON1 with several different inhibitors have been reported against paraoxon and phenylacetate substrates (Khersonsky and Tawfik, 2005).

A large number of structurally novel sulfonamide derivatives have recently been reported to show inhibitory activity towards different proteases (metallo-, serine, cysteines, or aspartic proteases of mammalian or viral origin), and hence substantial antitumor, anti-inflammatory, and antiviral activity. Although they have a common chemical motif of aromatic/heterocyclic sulfonamide, there are a variety of mechanisms of their biological action, some of them poorly understood at this moment (Supuran et al., 2003).

Sulfonamide, an important chemical used such as pharmacological agent for treatment, was found to be an inhibitor for human serum PON1 enzyme activity against both substrates under investigation. Esterase activity of the enzyme was more inhibited than its phosphotriesterase activity by sulfonamide. This suggests that both substrates are not hydrolyzed at the same active site, but it does obviously indicate that these substrates are positioned in the same manner. In addition, it appears that different substrates occupy different subsites within the same active site and make use of different catalytic residues (Harel et al., 2004).

Furthermore, sulfonamide was competetive inhibitor for human serum PON1 enzyme activity with paraoxon as substrate. It suggests that both sulfonamide and parao-

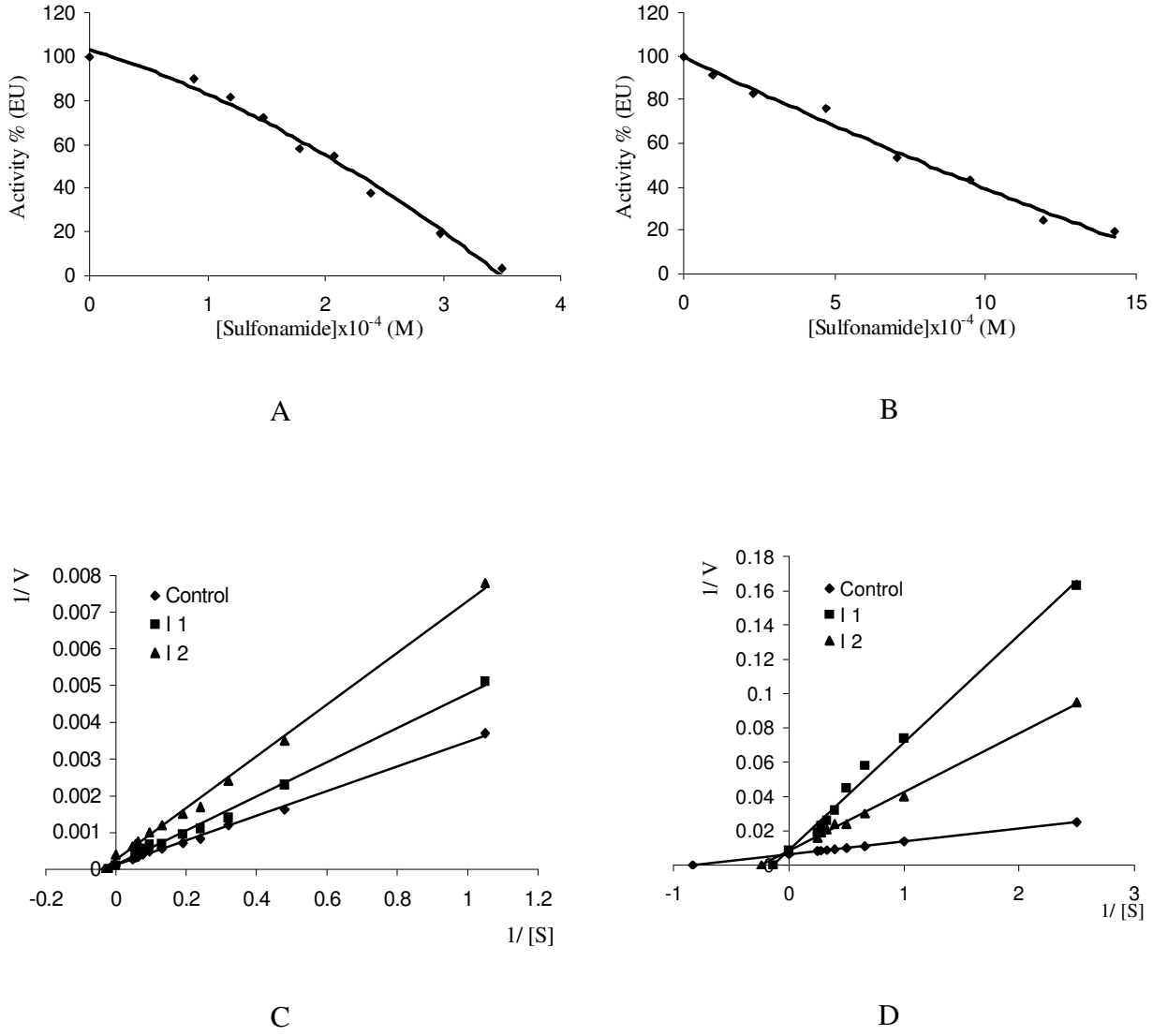

Figure 3. Inhibition of paraoxonase by sulfanilamide with and phenylacetate (A) and paraoxon (B) as substrat. A purified paraoxonase from human serum was assayed for paraoxonase and arylesterase activity in the presence on various concentrations of sulfanilamide.IC_{50} values of these were determined from A and B. The slope of Lineweaver-Burk plots indicates competitive inhibition for paraoxon (D) and non-competitive inhibition for phenylacetate (C).

xon bind the to same site of the enzyme. However, sulfonamid was a non-competitive inhibitor for the enzyme activity with phenylacetate as substrate. The differences between the effects of substrates are probably due to different modes of their binding (positioning, orientation) in the active site. The competetive inhibitory effect of 2-hydoxyquinoline on PON1 activity was also reported (Khersonsky and Tawfik, 2005; Aharoni et al., 2004).

As a conclusion, the aim of this study was to define the effects of sulfonamide on human serum PON1 with two subtrates and thus to evaluate the medical and side effects of this compund *in vitro*. Although sulfonamide being major component of some drugs and causes a positive effect on threatment of most illness, it dramatically inhibited the human serum PON1. This finding is considerable, because PON1 is one of the most important enzyme which has antioxidant and antitoxicological effects in organisms. Even though they are consumed in small amounts, stil can affect PON1. Consequently, the inappropriate use of sulfonamide or its derivatives potentially a risk to human healt and especially coronary patients.

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