Effects of acute *Staphylococcus aureus* infection on paraoxonase activity, thiol concentrations and ferric reducing ability of plasma in rabbits

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SUMMARY

The paraoxonase 1 (PON1) is recently considered as a new antioxidant enzyme, involving in inhibition of LDL oxidation and in inflammatory response in some species but not in rabbits. The aim of the study was to investigate the variations of the PON1 activity and of antioxidant systems globally assessed throughout the ferric reducing antioxidant power (FRAP) and the concentrations of low molecular thiol compounds (glutathione) in plasma from male White New Zealand rabbits (n = 7) experimentally subcutaneously infected with *Staphylococcus aureus* (highly virulent field strain, 8\(\times 10^8\) c.f.u./mL, 100 \(\mu\)L) for a 21 days long period after inoculation. Whereas the antioxidant markers remained stable in untreated control rabbits (n = 6), significant decreases in PON1 activity, FRAP values and thiol concentrations compared to the initial and control values were recorded on day 1, 2 and 3, respectively in infected rabbits and have persisted until the 7\(^{th}\) day for the enzyme activity, the 14\(^{th}\) day for the FRAP value and the 21\(^{st}\) day for the thiol concentrations. These results show that PON1 may be considered as a negative acute-phase protein in rabbits and that the acute bacterial *S. aureus* infection in rabbits is coupled to impairment in systemic antioxidant systems.

Keywords: rabbit, *Staphylococcus aureus*, acute bacterial infection, paraoxonase, ferric reducing ability of plasma, plasma thiol concentrations.

RESUME

La paraoxonase 1 (PON1) est une enzyme récemment considérée comme une enzyme anti-oxydante étant donné son implication dans l’inhibition de l’oxydation des LDL et dans la réponse inflammatoire. L’objectif de cette étude a été de déterminer les variations plasmatiques de l’activité PON1, du pouvoir réducteur du fer ferrique (PRFF) et des concentrations des composés thioles de faible poids moléculaire (glutatione) au cours du temps sur une période de 21 jours chez des lapins mâles blancs de Nouvelle Zélande (n = 7) inoculés par voie sous-cutanée par une suspension de *Staphylococcus aureus* (souche de terrain hautement virulente, 8\(\times 10^8\) c.f.u./mL, 100 \(\mu\)L). Alors que les 3 systèmes anti-oxydants sont restés stables chez les lapins témoins non traités (n = 6), des diminutions significatives par rapport aux valeurs de base et à celles obtenues chez les témoins ont été observées dès le 1\(^{er}\) jour pour l’activité PON1, le 2\(^{me}\) jour pour le PRFF et dès le 3\(^{me}\) jour pour les concentrations circulantes en thioles et les valeurs respectives de ces 3 paramètres sont restées faibles par rapport aux témoins jusqu’aux 7\(^{es}\)*, 14\(^{es}\)* et 21\(^{es}\)* jours. Ces résultats montrent que la PON1 pourrait être considérée comme une protéine négative de la phase aiguë de l’inflammation chez le lapin et qu’une infection bactérienne aiguë dans cette espèce est associée à une défaillance globale des capacités antioxidantes.

Mots-clés : lapin, *Staphylococcus aureus*, infection bactérienne aiguë, paraoxonase, capacité du plasma à réduire le fer ferrique, concentrations plasmatiques des composés thioles.

Introduction

*Staphylococcus aureus* is a Gram-positive bacterium that can cause a wide range of infections in humans and several animals through its toxin production. *S. aureus* is a prominent human pathogen and a leading cause of community- and hospital-acquired bacterial infections worldwide. In addition, in lactating animals, *S. aureus* is a common cause of chronic mastitis [11, 14]. One of the pathological consequences of *S. aureus* infection is the development of abscesses characterized by extensive recruitment of neutrophils and activation of their phagocytosis activity resulting in an oxidant/antioxidant imbalance [41, 42], considered as a hallmark for acute and chronic inflammation [17, 45].

The non-enzymatic antioxidant capacity of plasma is evaluated by several indexes and assays, such as 2,2-azinobis (3-ethyl-benzothiazolone-6-sulfonic acid) (ABTS, commercialized by Randox Laboratories as Trolox equivalent antioxidant capacity, TEAC assay), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), the oxygen radical absorption capacity (ORAC) and OXY-absorbent test [10, 38, 39, 57]. Each of these assays has advantages and disadvantages. FRAP values have been shown to be proportional to the reducing power of the main non-enzymatic antioxidants in the plasma, mainly uric acid [7, 40] and ascorbic acid [7], but does not detect reduced glutathione and other plasma thiols [57]. Thus measuring
both FRAP and the concentration of total thiols may allow an accurate determination of the state of the antioxidant defence systems in plasma.

In addition to non-enzyme antioxidants, the enzymes detoxifying ROS (reactive oxygen species) in plasma are pivotal in the global antioxidant status, too. Besides the well-known antioxidant enzymes such as superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPx), glutathione-S-transferases (GST), the plasma paraoxonase 1 (PON1) is suggested to be an important factor involved in inhibition of oxidation of LDL complexes. PON1 is a HDL-associated enzyme, belonging to the family of calcium-dependent hydrolases (esterases) that catalyzes the hydrolysis of many xenobiotics [12, 55]. Lately a new activity of PON1 has been established: a lactonase activity. It can hydrolyze a variety of aromatic and aliphatic lactones, including lactones of hydroxyl derivatives of arachidonic and docosahexaenoic acids and hydrolysates some oxidative products of the phospholipids, such as isoprostaten, carboxyl and aldehyde esters and hydroperoxides of the phosphatidylycholine, displaying phospholipase-A2-like activity [12, 15, 35]. That is the reason why it has been accepted that the lactones, hydroxy, hydroperoxy and oxidative derivatives of polyunsaturated fatty acids (PUFAs) most likely are the primary endogenous substrates of PON1 [1]. There is also a strong evidence that PON1 hydrolyzes also PAF (platelet-activating factor), one of the important pro-inflammatory mediators with phospholipid structure. It has been found that the all PAF-hydrolyzing activity of HDL is due to PON1 [35]. Most recently it has been reported that PON1 is active towards some other endogenous compounds, such as oestrogen esters [48]. Based on these observations, it has been considered that latter two enzyme activities, the lactonase and 3-esterase activities are the fundamental functions of PON1 contributing to its ability to prevent oxidative changes of both LDL and HDL particles and thus to decrease the risk of cardiovascular diseases [48]. The liver plays a key role in the synthesis of plasma PON1, however, the plasma PON1 activity is affected by a variety of genetic and non-genetic factors [8, 9, 12, 13, 21]. A cholesterol-rich diet given to wild-type rabbits and to rabbits transgenic for human apoa-I has been shown to result in a significant reduction of PON1 activity [34]. In humans, consumption of degraded cooking oil [46] lowered PON1 activity while alcohol [54] and vitamin C and E [27] elevated it. PON1 may also be altered as a part of the inflammatory response. In a study it has been shown that HDL complexes become pro-inflammatory factors during the acute phase response, possibly due to the loss of PON1 activity from HDL [56]. In another study it was described a decrease in serum PON1 activity and in hepatic PON1 mRNA in Syrian hamsters during the acute phase response induced by lipopolysaccharide (LPS) injection [19]. However, no such data in rabbits are available.

In this respect, the present study aims to assess the effect of acute Staphylococcus aureus infection in rabbits on the plasma antioxidant defence by measuring the concentration of total low-molecular thiols and the ferrous reducing antioxidant power (FRAP) in plasma as well as the plasma paraoxonase (PON1) activity.

**Material and Methods**

**ANIMALS AND PROTOCOL DESIGN**

The experimental procedure was approved by the Commission of Ethics at the Faculty of Veterinary Medicine of Trakia University, Stara Zagora, and during the entire study period the recommendations for caring and treatment of rabbits reared as experimental animals were followed.

In the current study, 13 male White New Zealand rabbits, 3 months old, weighing in average 3.2 ± 0.4 kg, were housed in grow out batteries with free access to water and commercial feed and were divided into 2 groups: 7 were infected with S. aureus by subcutaneous injection (100 μL) of a highly virulent field S. aureus strain suspension (8х10⁶ c.f.u./mL) as described by KLOOS and BANERMAN [31] and 6 rabbits were injected with saline, composing the control group.

Blood samples were collected by v. auricularis puncture into sterile microtubes containing heparin before injection, 6 hours after and at days 1, 2, 3, 7, 14 and 21. After centrifugation (1500 g, 10 minutes, 4°C), plasmas were stored at -20°C until assessed. Samples from the abscesses of the sick rabbits and samples from the internal organs of one died rabbit were cultivated on blood agar (BUL-BIO NCIPD, Sofia, Bulgaria) under aerobic conditions and temperature of 37°C for 24 hours.

**BIOCHEMICAL ANALYSES**

**Paraoxonase (PON1) activity**

The plasma PON1 activity was evaluated in the Biochemistry Laboratory in department of Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora, using an adapted by us method of TOMAS et al. [49]. The kinetic method is based on the velocity of hydrolysis of the substrate paraaxon by the enzyme leading to release of p-nitrophenol. Changes in the optical density (OD) at 405 nm were monitored for 5 minutes at 37°C and the plasma PON1 activity was expressed as U/L, one unit corresponding to 1 μmol of p-nitrophenol formed per minute. The molar extinction coefficient of p-nitrophenol is 18 053 M⁻¹ cm⁻¹ at pH 8.5.

**FRAP assay**

The plasma FRAP assay (ferric reducing antioxidant power or ferric reducing ability) was performed according to the method of BENZIE and STRAIN [7]. The method is based on the reduction of ferric (Fe³⁺) to ferrous (Fe²⁺) ion at low pH. This causes a formation of blue coloured ferrous-tripryldltriazine (Fe²⁺·TPTZ) complex, which absorbs
at 593 nm. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma [7, 32]. Aqueous solutions of known Fe²⁺ (FeSO₄, 7H₂O; Sigma Aldrich, USA) concentration, ranging from 0.2 to 1 mM were used for standard curve. The FRAP values (resulting Fe³⁺ concentrations) were expressed in mM.

**Plasma concentrations of thiol compounds**

The assessment of concentrations of thiols in plasma was performed by the adapted for microanalysis method of ELLMAN [6, 16]. The method is based on the interaction of reduced thiols (i.e. glutathione) with the reagent of Ellman [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB). The reduction of Ellman’s reagent by the thiol group leads to the 2-nitromercaptobenzoic acid anion, which has an intensive yellow colour and can be measured spectrophotometrically at 412 nm. Aqueous solutions of known GSH (SERVA Electrophoresis GmbH, Germany) concentration, ranging from 0.25 to 2 mM were used for standard curve. Results were expressed in mM.

**STATISTICAL ANALYSES**

Statistical analyses were performed using StatView v.4.53 for Windows (Abacus Concepts, Inc.). The ANOVA test was applied for comparing the continuous variables in independent groups and the paired t-test for comparison of the data in different periods after infection in each of the study groups. Factors with p < 0.05 were considered statistically significant.

**Results**

As shown in figure 1, no significant changes were observed in the plasma PON1 activity in control untreated rabbits and the baseline plasma PON1 activities were similar between the S. aureus infected and control animals (1680 ± 227 U/L and 1642 ± 107 U/L, respectively). In treated rabbits, the plasma PON1 activity has significantly declined (1115 ± 115 U/L) 24 hours after the bacterial inoculation compared to the baseline values (p < 0.05) and to values recorded in the control untreated rabbits (p < 0.01). The enzyme activity slightly increased at 48 hours (1302 ± 129 U/L) compared to 24 hours (48h vs. 24h; p < 0.05) and thereafter slowly and gradually continued to increase but remained depressed compared to the control values until the 7th day (p < 0.05). On days 14 and 21, the PON1 activities in plasma from infected animals became higher, although not significantly, than the baseline activity (1881 ± 161 U/L and 1875 ± 117 U/L on days 14 and 21, respectively).

The figures 2 and 3 present the variations of FRAP values and plasma thiol concentrations according to time in S. aureus inoculated and control rabbits. No statistically significant changes were detected in plasma FRAP values and in thiol concentrations in control untreated animals during the follow-up periods. No significant differences in the baseline values were recorded between infected and control animals. The FRAP variation profile in treated rabbits was similar to that of the paraoxonase activity except that the marked decrease of the FRAP values induced by S. aureus inoculation (0.712 ± 0.021 mM Fe³⁺) compared to the baseline (0.927 ± 0.044 mM Fe³⁺, p < 0.001) and control values (0.894 ± 0.115 mM Fe³⁺, p < 0.001) was observed at 48 hours instead of at 24 hours, and thereafter, this parameter slowly and gradually increased but remained significantly depressed compared to the baseline values at 72 hours (p < 0.01) and to the control values at 72 hours and on day 14 (p < 0.05) (figure 2). By contrast, the kinetics of plasma low molecular thiol concentrations in infected rabbits exhibited a quite different pattern than the 2 other parameters (PON1 activity and FRAP value): the significant decline in thiol concentrations to the baseline values at 72 hours (0.419 ± 0.052 mM GSH, p < 0.01) and control (0.400 ± 0.103 mM GSH, p < 0.05) values was delayed and noted only on day 3 (0.712 ± 0.021 mM GSH) but was markedly prolonged until the 7th day compared to the controls (p < 0.05) and until the 21st day.
compared to the initial values (on day 7: p < 0.001, on day 14: p < 0.01 and on day 21: p = 0.001) (figure 3).

**Figure 3:** Variations in plasma low molecular thiol concentrations according to time in rabbits after S. aureus (8x10^6 c.f.u./mL) subcutaneous inoculation (100 µL) (treated, n = 7) or after saline injection (controls, n = 6). Results are expressed as mean ± standard deviation. Treated vs. controls: a: p = 0.05 and b: p < 0.05; for the treated group: c: p < 0.01 for 0 h vs. 72 h and day 14; d: p < 0.001 for 0 h vs. day 7; e: p = 0.001 for 0 h vs. day 21.

**Discussion**

The major finding in this study is that the acute *Staphylococcus aureus* infection caused a marked decrease in plasma antioxidant defence systems illustrated by significant reductions in plasma paraoxonase activity, in plasma FRAP and thiol concentrations in infected rabbits. These observations are in agreement with the generally accepted concept that inflammation is accompanied by increased production of ROS mainly due to respiratory burst in activated macrophages resulting in local and systemic oxidative stress [37]. Similar decreases in serum thiol concentrations and in paraoxonase activity were reported for patients infected with *Mycobacterium tuberculosis* suffering from acute pulmonary tuberculosis [43], in patients with HCV (hepatitis C virus) infection in haemodialysis [26] and in patients with chronic hepatitis [2]. Interestingly, the plasma paraoxonase activity in rabbits was extremely higher than in humans [18], as also shown by CABANA et al. [9]. Changes in paraoxonase PON1 activity has been studied in different physiological conditions of dairy cows, such as pregnancy and early postpartum period and it has been reported that significant decreases in the paraoxonase activity occurred in dry cows compared to lactating cows [51], in the early postpartum period compared to the late non-pregnant lactation [50], as well as in early and late puerperium than in mid-lactation [52]. The observed low PON1 activity in dry period and early postpartum was accompanied by increased MDA concentrations, indicating the existence of prooxidant/antioxidant imbalance influence by reproductive stress. In another study with periparturient dairy cows it was demonstrated that plasma paraoxonase activity correlated positively with retinol binding protein and albumin (negative acute phase proteins) and negatively with haptoglobin (positive acute phase protein) [8]. Thus PON1 activity was suggested to serve as an index of liver function and to be considered as a negative acute phase protein [8]. Earlier, it was demonstrated by FEINGOLD et al. [19] that serum PON activity and liver mRNA PON1 synthesis in Syrian hamsters was significantly decreased during the acute phase response after LPS injection and these authors firstly proposed the role of PON1 as a negative acute phase protein.

In addition to studies focused on the changes of paraoxonase activity in different human diseases, there are several works exploring the systemic oxidative stress in patients with different parasitic [29, 36], viral [20, 24] and gram-negative infectious diseases [30, 33, 37, 44]. However, little is known about the relation between gram-positive bacteria, such as *Staphylococcus aureus* and oxidative stress in humans. The exposure of human monocytes and macrophages to *S. aureus* has been shown to induce a selective stress response consisted of increased SOD activity, synthesis of some heat-shock proteins and *de novo* synthesis of heme oxygenase [28, 47].

In studies with infected animals decreases in blood antioxidant capacity and in antioxidant concentrations have been reported for neonate rats after meningitis caused by *Streptococcus agalactiae* [5], mice infected with influenza A virus [25], for BALB/c mice with tularemia on day 5 post-infection [4], and for European brown hares on day 2 after the experimental infection with *Francella tularensis* [3]. So far, we have not found in the literature data concerning the effect of experimental infection with *Staphylococcus aureus* on the systemic antioxidants in any animal species. There was only a report for an increase in SOD (superoxide dismutase) activity, decrease in GPx (glutathione peroxidase) activity and no changes of CAT (catalase) activity, and accumulation of lipid peroxidative products (MDA) in mucosa of experimentally induced *S. aureus* infection of sinuses in rabbits [53]. To the best of our knowledge this is the first study demonstrating the effect of *Staphylococcus aureus* infection on the antioxidant capacity of plasma and some antioxidants, particularly low-molecular thiols and PON1 not only in rabbits but in any other experimental animal species.

In the present study a significant decrease in plasma paraoxonase activity was also observed compared to the baseline value and to the control group 24 hours after *S. aureus* inoculation in rabbits. Previously results obtained in our laboratory indicated similar changes of albumin concentrations and significant increase in plasma fibrinogen concentrations during experimentally induced *E. coli* infection in weaning rabbits [23]. Recently, an increase in plasma haptoglobin concentrations was also reported in obese rabbits infected with *S. aureus* [22]. Taken together the previous findings and the present results strongly suggest that PON1 may be considered as a negative acute phase protein also in rabbits during acute inflammation with gram-positive bacteria, such as *S. aureus*. In addition, it was confirmed that the acute bacterial *S. aureus* infection in rabbits is accompanied by impairment in systemic antioxidant defence systems most possibly resulting from the depletion of non-

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**VLAYKOVA (T.) AND COLLABORATORS**
enzyme antioxidants in the condition of high activation of neutrophils and increased ROS generation during the phagocytosis.

REFERENCES


