Is there an unidentified defence mechanism against infection in human plasma?

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Abstract The total peroxyl radical scavenging capacity (TRAP) of human plasma was measured from pneumonia patients and controls. TRAP and its main components, ascorbic acid, alpha-tocopherol, uric acid or protein thiol groups, were unaltered, but the concentration of unidentified antioxidants in pneumonia patients was significantly reduced. Our results indicate that human plasma may contain so far unidentified antioxidants depleted in infection.

Key words: Antioxidants; Unidentified antioxidants; Infection; Ascorbic acid

1. Introduction

It is commonly assumed that an effective defence against bacterial infection requires release of active oxygen species during the neutrophil respiratory burst. This may play an important role in causing damage to host tissues [1]. The polymorphonuclear neutrophil (PMN) has a complex enzyme system that reduces oxygen to superoxide ($O_2^{\cdot-}$) [2]. Both $O_2^{\cdot-}$ and H_2O_2 can be detected after the PMN is activated and a substantial fraction of H_2O_2 is consumed by myeloperoxidase to produce hypochlorous acid (HOCl) [3]. HOCl is a potent oxidant which reacts readily with biological molecules. It is cytotoxic to a wide variety of mammalian cells [4]. Since HOCl can account for at least 40% of the $O_2^{\cdot-}$ generated during the respiratory burst [5], it may be responsible for most of the oxidant damage mediated by neutrophils [6].

In plasma, stimulated polymorphonuclear leukocytes initiate very rapid oxidation of ascorbate, followed by partial depletion of urate [7]. It has been proposed that plasma thiol-groups (which are known to be largely located on albumin) are in quantitative terms the most important scavengers of HOCl [8]. However, if extra ascorbate is added to plasma, ascorbic acid becomes the most important scavenger of HOCl [8]. Plasma ascorbic acid is known to decrease in acute pancreatitis and other intra-abdominal crises [9], and urine ascorbic acid concentration decreases during a common cold [10].This study set out to explore the effect of acute infection

upon plasma antioxidant defences in a case-control design involving hospitalized pneumonia patients.

2. Materials and methods

Sixteen hospitalized pneumonia patients were studied from the Department of Infectious Diseases of Tampere City Hospital. Sixteen controls were matched by age, gender and other disease. The mean age of the patients (10 male and 22 female) was 66.4 years (range 33–85), and the mean age of the controls (10 male and 22 female) was 65.8 (range 33–85 years). The diagnosis of pneumonia was based on clinical symptoms and laboratory evidence of infection, increased CRP and ESR, a new or progressive infiltrate on the chest X-ray and response to antibiotic therapy. Urine creatinine clearance was normal in all patients. The pneumonia patients received penicillin G (1–2 mill. IU four times/day intravenously) or cefuroxime (750 mg–1.5 g three times/day intravenously). None of them was given O_2 treatment during the experiment. The diet was standardized hospital food.

2.1. The plasma sample

All blood samples were collected within 1–3 days (mean 2.3) of the diagnosis of pneumonia. The samples of venous blood were drawn from the antecubital vein into precooled EDTA-containing Vacutainer tubes. The samples were placed in an ice bath, protected from light and immediately transported to the laboratory where the plasma was separated by centrifugation. The plasma samples were stored in liquid nitrogen until analyzed. For ascorbic acid determination, a special tube containing meta-phosphoric acid (5% final concentration) was prepared.

Total peroxyl antioxidant capacity (TRAP) was determined by the chemiluminescence method, which is described in full detail elsewhere [11]. Briefly, constant production of peroxyl radicals by ABAP (2,2′-azobis [2-amidinopropane] hydrochloride) is followed by luminol-enhanced chemiluminescence. The time for which the added test sample extinguishes the reaction is directly proportional to the peroxyl radical-trapping antioxidant capacity of the sample [11,12]. TRAP is thus expressed as micromoles of peroxyl radicals trapped by one litre of the sample.

In addition to direct measurement of TRAP (TRAPmeas), calculated TRAP (TRAPcalc) was derived from the concentrations of individually measured antioxidants in plasma with experimentally determined stoichiometric factors [11,12]: For protein SH-groups the stoichiometric factor was 0.4, for ascorbic acid 0.7, for uric acid and for tocopherol 2.0. Thus TRAPcalc = 2.0×(tocopherol concentration) + 2.0× (uric acid) + 0.7× (ascorbic acid) + 0.4×(SH-groups). The percentage contribution of individual components was calculated from total TRAPmeas. The difference between measured TRAP and calculated TRAP (TRAPmeas − TRAPcalc = UNID) represents the antioxidative capacity of as yet unidentified antioxidants.

For the evaluation of the method of analysis, artificial plasma was prepared by mixing the known antioxidants of plasma (urate, -SH groups of GSH, ascorbic acid and Trolox) in different concentrations with the assay buffer as described elsewhere [13]. The correlation between measured and calculated TRAP was linear, as shown earlier [13], especially in cases where the theoretical TRAP was similar to that of human plasma (>700 μmol/l). This strongly suggests not only that the stoichiometric factors are highly accurate, but also that there is no recycling or regeneration of the components.

The concentrations of uric acid and ascorbic acid were measured by

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HPLC with an electrochemical detector according to the method of Frei et al. [14]. Alpha-tocopherol was determined by the modified HPLC method [15]. The protein SH-groups were determined according to Ellman [16].

The method of whole blood chemiluminescence (CL) has been described earlier [17]. Briefly, cold suspensions of reagents and anticoagulated blood (EDTA) were mixed. CL emission was measured at 37°C for 60 min in a 500 µl HBSS buffer containing 4×10^{-4} M luminol (Sigma), 0.1% gelatin, 1 mg of opsonized zymosan (Sigma) and 50, 100 or 200 nanoliters of blood, using an automated luminometer setup (LKB Wallac 1251 Luminometer connected to a PC). Maximum CL, (usually obtained 25 min after reaction onset) was plotted against each blood sample volume and expressed as mV/µl.

2.2. Statistics

Data analysis is based on analysis of variance and analysis of variance for repeated measures. Multiple regression analysis and Student's t-test were also used. All results are given as means±S.E.M.

The study protocol was approved by the Ethical Committee of Tampere City Hospital.

3. Results

The total peroxyl radical scavenging capacity of plasma (TRAP) decreased in a non-significant way in the patient group. Among the known components of TRAP (uric acid, ascorbic acid, tocopherol, SH-groups) no difference was detected in mean plasma concentrations. However, a highly significant ($P < 0.0001$) difference was found in the amount of unidentified antioxidants between the patient and control group (299 ± 30.8 µmol/l versus 506 ± 25.8 µmol/l). All the plasma concentrations are shown in Table 1.

As an indicator of infection, the chemiluminescence (CL) produced by polymorphonuclear leukocytes (PMN) was significantly ($P = 0.0186$) higher in the patient group, 180.2 ± 34.4 mV/µl, compared with the control value, 108.8 ± 6.5 mV/µl. The mean leukocyte concentration was 7.01 ± 0.67 E9/l in the patient group and 5.89 ± 0.65 E9/l in the control group.

4. Discussion

The purpose of this study was to follow the changes occurring in the total peroxyl scavenging capacity of human plasma in a state of increased radical production (e.g. acute infection).

The most important finding with regard to plasma antioxidant defences was the sharp decrease in the amount of unidentified antioxidants of human plasma in the patient group. No other significant changes were seen in plasma antioxidant values, although the TRAP concentration in the patient group was lower than in the controls. Uric acid increased non-significantly in the patient group, probably because of ischemia. Previous results indicate that an increase in uric acid concentration results in increased TRAP concentration, without this affecting the amount of unidentified antioxidants [18]. Vitamin C levels were low in both groups. Low vitamin C intake is a typical finding in the Finnish population, especially in males and older age groups [19].

Several earlier studies attest to the usefulness of TRAP in evaluating extracellular fluid antioxidant ability [13,18,20]. Among the different components of TRAP, the proportion of unidentified antioxidants in human plasma in this study was 28% in the patient group and 42% in the control group. The latter figure is consistent with earlier findings [13,18]. There are different ways in which the observed existence of unidentified antioxidants in human plasma can be explained. One possible explanation lies in the methodology applied. Stoichiometric factors used for the identified components of TRAP could be too low, or recycling or regeneration of the components could occur. However, considering our results with artificial plasma, we have shown that this is not the case [13,21]. The mean difference between TRAP measured and calculated by the stoichiometric factors for individual components was only about 80 µmol/l, which indicates that not only are the factors very precise, but also that there is no recycling or regeneration of the components. Our earlier result clearly showed that the stoichiometric factor of ascorbic acids (AA) is concentration-dependent [13]. In cerebrospinal fluid (CSF), where the AA concentration is 8–10× higher than in plasma, the stoichiometric factor was three times lower [13]. Earlier studies have drawn attention to similar phenomena, indicating a concentration-dependent radical trapping capability in ascorbate [14,20]. However, in concentrations occurring in plasma the stoichiometric factor is in the range of 0.6–0.8 [11,13].

Other compounds such as bilirubin, β-carotene and flavonoids have been proposed to act as antioxidants in vivo and

Table 1
Plasma mean concentrations (µmol/l) of TRAP and its components in the patient and control group

	Group	Mean ± S.E.	n
TRAP	patients	1065 ± 71.8	16
	controls	1201 ± 42.8	16
Uric acid	patients	278 ± 25.5	16
	controls	242 ± 17.7	16
SH-groups	patients	391 ± 19.6	16
	controls	381 ± 19.9	16
Ascorbic acid	patients	32 ± 2.9	16
	controls	33 ± 4.7	16
Alpha-tocopherol	patients	15 ± 1.2	16
	controls	17 ± 1.8	16
UNID	patients	299 ± 30.8***	16
	controls	506 ± 25.5	16

UNID = unidentified antioxidants, ***$P < 0.0001$.

could be one part of UNID. However, Lindeman et al. [22] have shown that by recalculating the proportion of bilirubin from TRAPcalc (using a stoichiometric factor of 2.0) its percentage contribution is less than 4%. Also, the plasma concentration of β-carotene is too low to make a significant contribution to TRAPcalc [22]. Our preliminary data show that flavonoids have a minor contribution (less than 1%) to TRAP (Metsä-Ketelä et al. unpublished data) and they cannot be responsible for the large proportion of UNID. We propose that human plasma contains one or more important peroxyl radical trapping antioxidants that as yet remain unidentified. Earlier results from our laboratory indicate that this UNID may be induced by oxidative stress such as severe preeclampsia [18].

The patients in this study were treated with either penicillin G or cefuroxime. Earlier data suggest that cephalosporines, ampicillin and piperacillin are capable of inhibiting the neutrophil-mediated lysis of target cells, while penicillin G is ineffective [23]. Cephalosporines are proposed to be scavengers of HOCl at therapeutically relevant concentrations [24]. However, in our study the patient group showed a significant decrease in the amount of unidentified antioxidants, and the possible beneficial effect of cephalosporines did not protect the antioxidant defence system against the HOCl increase.

This study showed that in acute infection the amount of classical antioxidants remains unchanged, whereas the amount of unidentified antioxidants decreases.

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