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Unidentified Antioxidant Defences of Human Plasma in Immobilized Patients: A Possible Relation to Basic Metabolic Rate

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Plasma total peroxyl radical scavenging capacity was studied in terminal patients who were chronically immobilized because of an acute (stroke) or chronic neurodegenerative disease (Alzheimer's disease). A luminometric assay was used to measure total antioxidant capacity (TRAP). The immobilized patients showed significant decrease in TRAP primarily because of a decrease in the concentration of unknown antioxidants. Our results suggest that human plasma may contain unknown antioxidants, the regulation of which could be related to the basic metabolic rate.

Keywords: Antioxidants, unidentified antioxidants, TRAP, immobilization, basic metabolic rate

Abbreviations: TRAP, total peroxyl radical scavenging capacity; AA, ascorbic acid; SH-groups, sulfhydryl-groups; UNID, unidentified antioxidants; AD, Alzheimer's disease; ABAP, 2, 2'-azo-bis (2 amidinopropane).

INTRODUCTION

Possibly related to basic metabolism, the formation of free radicals and other reactive oxygen species is a normal consequence of oxidative reactions in the body.[1] The inverse relationship between longevity and metabolic rate appears to vary between different groups of mammals. This may be related to differences in mitochondrial $O_2^{\bullet-}$ and H_2O_2 generation.[2] Mitochondria from shorter-lived species produce higher amounts of reactive oxygen species than do those from longer-lived species.[2] However, there are species (such as pigeons) in which the extraordinarily long mean life span cannot be explained by their basal metabolic rate, because their mitochondria show a lower free radical leak in the respiratory chain.[3] They may show low levels of antioxidants because their rates of free radicals are low, whereas the high rates of free radical production of short-lived species (such as the rat) are compensated by high levels of endogenous antioxidants.[3]

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The aim of this study was to estimate the effects of immobilization—a decrease in basic metabolic rate—on the total peroxyl radical trapping capacity of human plasma and its main components, ascorbate, alpha-tocopherol, uric acid, protein sulfhydryl (SH)-groups and the fraction of unknown antioxidants (UNID). Two groups of patients, suffering from acute (stroke) or chronic (Alzheimer's disease, AD) degenerative brain disease, were followed-up for two years or until death. All the patients were immobilized and in the terminal stage of their disease. The controls were healthy age-matched volunteers and ambulatory patients in the early stages of AD.

MATERIALS AND METHODS

Two groups of immobilized patients from Tampere City Hospital were followed-up for two years or until death. The first group consisted of 13 women with Alzheimer's disease (AD-I); the second group consisted of 14 stroke patients (ST-I). In AD-I the diagnosis of AD was based on the clinical course of the disease. Patients with any additional diseases were excluded. The diet was standardized hospital food, none of the patients had parenteral nutrition. All the patients and controls were non-smokers. In AD-I the mean age was 85.5 years and the mean duration of immobilization 3.7 years; and in ST-I 85.1 years and 3.9 years, respectively. In both groups all the patients received anti-inflammatory analgesics (naproxen 250–500mg/day), neuroleptic drugs (melperone 25–50 mg/day and haloperidol 0.5–2.0 mg/day) and laxatives. In the stroke group four patients received digitalis (62.5 μg/day) or isosorbide dinitrate (20–40 mg/day). The control groups consisted of 14 healthy elderly (H-C) (mean age 84.5 years) and nine ambulatory patients with AD (AD-C) (mean age 65.5 years, range 59–81), who lived at home and used no prescription drugs or vitamins. Diagnosis of the AD-C group was based on the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke—Alzheimer Disease and Related Disorders Association) criteria, and The Clinical Dementia Rating Scale was used to assess the severity of dementia. These patients were examined at the Department of Neurology, Tampere University Hospital.

The plasma samples from the immobilized patients were taken four times a year between April 1992 and June 1994. Only one sample was collected from healthy controls and ambulatory AD patients.

The samples of venous blood were always drawn at the same time of the day 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 at –70°C until analyzed within two months. For ascorbic acid determination, a special tube containing metaphosphoric acid (5% final concentration) was prepared.

Chemicals and Instruments

ABAP (2,2'-azo-bis (2-amidinopropane) hydrochloride) was purchased from Polysciences (Warrington, PA, USA) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) from Sigma (St Louis, MO, USA); TROLOX (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxyl acid) was a generous gift from F. Hoffman-La Roche Ltd (Basel, Switzerland). A Pharmacia LKB Wallac Luminometer 1251 was used for measuring chemiluminescence. The luminometer was controlled and the data collected and processed by a PC computer. The program was purchased from TriStar Enterprise Oy (Tampere, Finland).

Determination of Trap

The principles of TRAP determination and our modification of the method are described in

greater detail in.[4–9] Briefly: Peroxyl radicals are produced at a constant rate by thermal decomposition of ABAP. Peroxyl radical reactions are followed by luminol-enhanced chemiluminescence. The time for which the added test sample of plasma extinguishes the reaction is directly proportional to the peroxyl radical-trapping antioxidative capacity of the sample, i.e. TRAP, which is expressed as micromoles of peroxyl radicals trapped by one liter of the sample.

The reaction was initiated by mixing 450 µl of oxygen-saturated sodium phosphate buffered (100 µM, pH 7.4) saline with 50 µl of 400 mM ABAP (prepared in 100 mM phosphate-buffered saline). The cuvette was placed in the temperature-controlled sample carousel of the luminometer (37°C). The computer automatically measured the chemiluminescence of each cuvette at intervals of about 36 sec. At the same time, the computer read the time for measurement. The sample was injected at 15 min, when the chemiluminenscence was already stabilized. The sample or standard, in a volume of 20 µl, was injected directly into the cuvette and the computer continued measuring the chemiluminescence at the same interval.

A water-soluble tocopherol, TROLOX, which is known to trap two radicals per molecule[5] (i.e. its stoichiometric factor is 2.0), was used as a standard. Both intra- and interassay variations of the method are 2%.[8] The TRAP of plasma was not changed within two months if the samples were kept at –70 C.

In addition to the direct measurement of TRAP, a calculated TRAP (TRAPcalc) was also derived from the concentrations of individual peroxyl radical-trapping antioxidants in plasma with experimentally determined stoichiometric factors.[8,9] These factors (n) indicate the molar amount of peroxyl radicals trapped by each mol of antioxidants. When these antioxidants were tested in the chemiluminescent reaction and compared with TROLOX, the following stoichiometric factors were obtained: protein SH-groups 0.4, ascorbic acid 0.7 and uric acid 2.0. Thus TRAPcalc = 2.0 (tocopherol) + 2.0 (uric acid) + 0.7 (ascorbic acid) + 0.4 (SH-groups). The difference between the measured TRAP and calculated TRAP (TRAPmeas – TRAPcalc = TRAPdelta) is composed of the antioxidative capacity of as yet unidentified antioxidants.

For evaluation of the analytical system, artificial "plasma" was prepared in phosphate (100 µM) buffered saline (pH 7.4) by solving the major known antioxidants of human plasma in the following concentration ranges: urate 125, 250, 500 µM, SH-groups (as reduced glutathione) 250, 500, 100 µM, Trolox C (as vitamin E) 25, 50, 100 µM and ascorbate 50, 100, 200 µM. For each combination both an experimental and a theoretical value was determined.

Other Chemical Determinations

The concentrations of uric acid and ascorbic acid were measured by HPLC with an electrochemical detector according to the method of Frei et al.[10]

Alpha-tocopherol was determined by the modified HPLC method of Catingnani et al.[11] In our modification the UV detection of antioxidants was replaced by an LC-4 amperometric detector (Bioanalytical Systems Inc., West Lafayette, USA). The applied potential was +1.0 V. The protein SH-groups were determined according to Ellman.[12]

The blood tests were taken with the patients' or relatives' permission. The study protocol was approved by the Ethical Committee of Tampere City Hospital and Tampere University Hospital.

RESULTS

There were no differences in any of the measured parameters between the immobilized Alzheimer (AD-I) and stroke (ST-I) patients or between the healthy controls (H-C) and ambulatory Alzheimer (AD-C) patients. In the healthy control group the percentage contributions of the different components to TRAP were 46% for uric acid, 2.3% for

ascorbate, 2.6% for tocopherol and 14.9% for SH-groups. A significant decrease ($p = 0.0001$) in TRAP concentration was observed due to immobilization when compared to the control groups (H-C 1217.6 ± 48.5 μmol/l and AD-C 1237.3 ± 64.2 μmol/l versus 877.8 ± 50.4 μmol/l in the AD-I group and 884.8 ± 48.5 μmol/l in the ST-I group; *see Fig. 1*). In immobilized patients a significant decrease was seen in uric acid, ascorbate and tocopherol (Table I), but not in SH-group concentration. In addition, there was a highly significant ($p = 0.0001$) decrease in the fraction of unidentified antioxidants in the immobilized patients (H-C 394.6 ± 36.7 and AD-C 417.6 ± 48.6 versus 232.9 ± 38.1 in the AD-I group and 182.3 ± 36.7 μmol/l in the ST-I group) (Fig. 1) During the two-year follow-up study of immobilized patients, no significant changes were observed in TRAP values. No correlation was found between the duration of immobilization and TRAP values.

DISCUSSION

In the present study plasma total peroxyl radical-trapping capacity was assessed by measuring the ability of a plasma sample to quench peroxyl radicals generated at a constant rate. TRAP measures the total peroxyl radical-trapping capacity. Virtually all chain-breaking antioxidants are included, but the preventive antioxidants are excluded. The capacity of a plasma sample to trap other non-water-soluble biologically relevant radicals cannot be derived from its TRAP value.[9,13] Another disadvantage of the TRAP method is that it gives only quantitative results but does not measure the reactivity of antioxidants in the sample. Furthermore, the effect of free radical scavenging upon luminescence may be due to trapping of peroxyl radicals or the luminol derived radicals.[14] However, when this was tested by the alpha-tocopherol analogue Trolox, which is a competitive quencher of human plasma, at higher concentrations of Trolox trapping of peroxyl radicals constituted the main process leading to the observed luminescence quenching. The trapping of luminol derived radicals may be more important in fluids with very low free radical scavenging concentrations, not in human plasma.[14] In spite of its limitations, TRAP has been proved to be a useful method for studying the net effect of oxidative load on the peroxyl scavenging antioxidants of human extracellular fluids.[7,8,9,13]

The most striking finding with regard to plasma antioxidant capacity was the significant decrease in TRAP in both immobilized groups, mostly resulting from the decrease in unidentified antioxidants (UNID). In this study UNID accounted for 36% of total TRAP in the AD-control and 22% in the immobilized (AD-I) group. Previous results from our laboratory indicate that in plasma peroxyl radical trapping capacity there is an unknown component, which reacts independently from the known components of TRAP.[15] The concentration of the well-known plasma antioxidants, bilirubin, β-carotene or flavonoids is too low[5,16] (Metsä-Ketelä, unpublished data) to explain the large proportion of UNID. Micromolar concentrations of hydroperoxides are formed during ABAP-initiated plasma oxidation once ascorbate is completely consumed.[17] These lipids are assumed to have a stoichiometric factor of 1.0 because during the phase of inhibited lipid peroxidation each molecule of oxidized lipid has reacted with one peroxyl radical.[17] There is evidence for abnormal levels of malondialdehyde and lipid peroxides in the brains, but not in serum of patients with AD.[18] The decreased rate of lipid peroxide formation following decreased Basic Metabolic Rate could partly explain the results in this study, but this is not consistent with our previous findings.[15,19]

Significant changes were observed in ascorbic acid, tocopherol and urate concentrations in the immobilized patients, but not in the ambulatory AD patients. In previous studies elevated superoxide dismutase levels have been detected in AD[20] and changes have also been reported in levels of alpha-tocopherol and glutathione in the

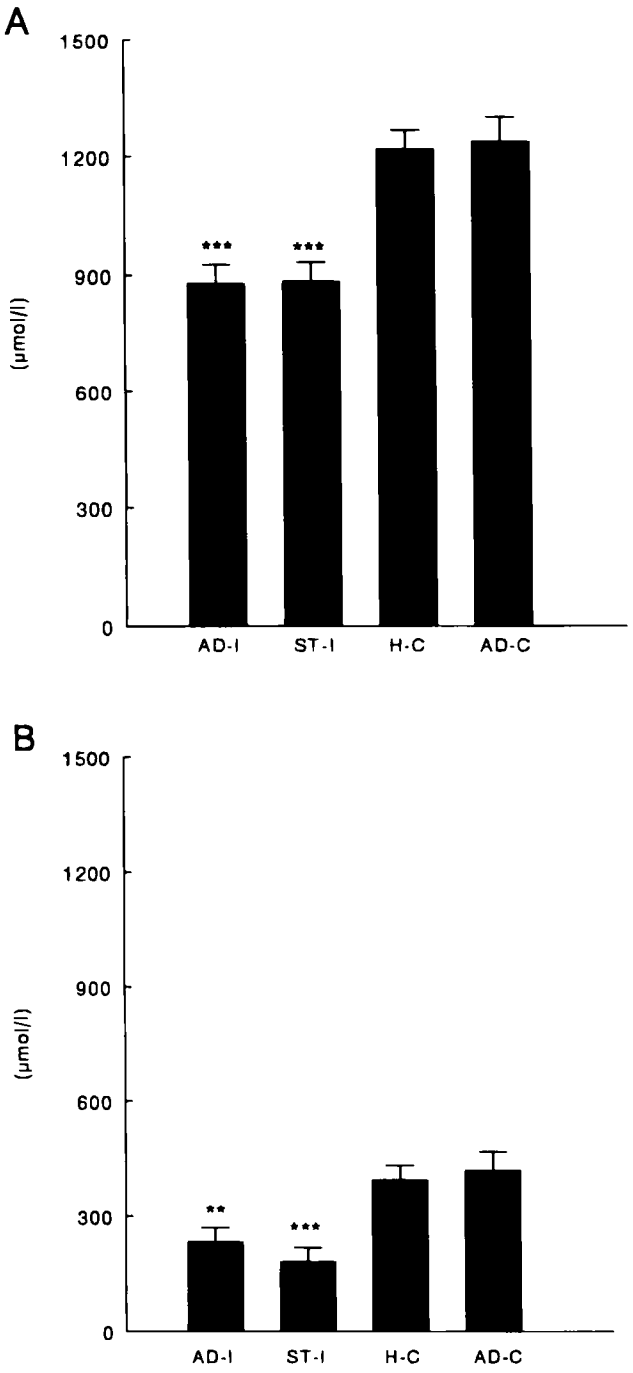

FIGURE 1 In A the plasma concentrations (µmol/l) of total peroxyl radical trapping capacity (TRAP) and in B the contribution of the unidentified antioxidants (TRAP-U) in healthy (H-C, n = 14), ambulatory AD (AD-C, n = 9), immobilized AD (AD-I, n = 13) and stroke (ST-I, n = 14) patients.

TABLE I The concentrations of the known components of TRAP, ascorbate, alpha-tocopherol, uric acid and protein SH-groups (umol/l) in immobilized patients (AD-I, ST-I) and controls (H-C, AD-C).

	H-C (n = 14)	AD-C (n = 9)	AD-I (n = 13)	ST-I (n = 14)
URIC ACID	286.6 ± 16.5	264.8 ± 21.8	210.8 ± 17.1**	239.2 ± 16.5*
ASCORBATE	40.6 ± 3.3	34.1 ± 4.4	21.7 ± 3.5***	17.0 ± 3.3***
TOCOPHEROL	22.2 ± 1.8	24.8 ± 2.4	15.1 ± 1.9**	14.8 ± 1.8**
SH-GROUPS	442.4 ± 31.8	541.6 ± 42.1	449.9 ± 33.0	456.5 ± 31.8

*p < 0.05, **p < 0.01, ***p < 0.001 compared with healthy controls (H-C).

brains of AD patients.[21] A decrease in glutathione peroxidase and SOD activity in erythrocytes and vitamin E and C deficiency in plasma has been found in AD patients, but most of these findings have come from a malnourished subgroup of patients.[22] In this material plasma vitamin concentrations were low, which is a common finding in the Finnish population, especially among old age groups.[23] Previous results indicate that almost half of the nursing home residents have dietary intakes of water-soluble vitamins below the minimum requirements.[24] Decreased content of lipids in the plasma of immobilized patients could partly explain the difference in alpha-tocopherol between the immobilized patients and the controls.

The vitamin deficiencies found in this material could indicate a nutritional origin for UNID. This is not, however, confirmed by other results from our laboratory. We have observed elevated plasma levels of UNID during a heavy physiological oxidative load caused by physical exertion (Metsä-Ketelä, unpublished data). Pathological stress caused by a serious infection (pneumonia) also leads to a significant decrease in UNID.[15] We have also found that in patients with small lung cell cancer, the first adriamycin injection leads to an increase in UNID within 20 hours.[19] In this study long-term immobilization supposedly caused a clear decline in UNID. Increased oxidative stress caused by the underlying disease instead of immobilization could be the reason for declined antioxidant defences. However, we had a control group of ambulatory AD patients living at home, and no significant changes could be found between the home-living AD patients and the healthy controls. Immobilized patients may also have more advanced diseases and, therefore, more oxidative damage, but the patients were followed for two years and in that time no significant changes in TRAP or its components was found. These observations indicate, that UNID may be related to basal metabolism.

It is also unlikely that the difference between immobilized patients and controls could be explained by medication. Some anti-inflammatory drugs (such as diclofenac) have peroxyl radical scavenging activity[25] and some neuroleptic drugs (such as chlorpromazine), but not haloperidol, are scavengers of organic peroxyl radicals.[26] Haloperidol has hypochlorus acid scavenging capacity.[26] However, these drugs may have a counteracting effect on oxidative stress rather than depleting antioxidant defences.

In conclusion, the main finding of this study was that the immobilized patient group, with a decreased basic metabolic rate, showed a decreased in unidentified antioxidants.

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