Clinica Chimica Acta 393 (2008) 114–118

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Modification of the oxidative stress biomarker AOPP assay: Application in uremic samples

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ARTICLE INFO

Article history:
Received 24 January 2008
Received in revised form 19 March 2008
Accepted 19 March 2008
Available online 1 April 2008

Keywords:
Advanced oxidation protein products
Triglycerides
Chronic kidney disease
Oxidative stress
Inflammation

ABSTRACT

Background: The levels of advanced oxidation protein products (AOPP), a plasma protein biomarker used to assess oxidative stress, are elevated in patients with chronic kidney disease (CKD). However, this apparent elevation is to a large extent due to assay interference (mostly by triglycerides which are usually markedly elevated in CKD). We therefore developed and tested a modified version of the AOPP assay to minimize the impact of this interference.

Methods: Plasma levels of AOPP, lipids, proteins and various biomarkers of inflammation and oxidative stress were analyzed in 218 prevalent hemodialysis patients and 13 healthy controls using the established original (*oAOPP*) assay and following precipitation of plasma lipids using dextran sulphate (modified assay, *mAOPP*). The modified results were validated against a lipid extraction procedure using ether/butanol.

Results: The modified assay decreased the levels of triglycerides and AOPP by 87% and 38%, respectively. Whereas oAOPP values correlated strongly with triglycerides, no such correlation was seen with mAOPP. The mAOPP levels correlated significantly with the oxidative stress markers 8-oxo-dG and pentosidine, whereas no such correlations were found for oAOPP.

Conclusions: The oAOPP concentration is largely overestimated in plasma samples due to lipid interferences. Precipitation of triglycerides before analysis yields markedly lower mAOPP values which more accurately reflect oxidative stress. Based on these results we propose that AOPP should be analyzed using the modified assay, which is a cheap, simple and fast method.

1. Introduction

The unfriendly uremic milieu is associated with increased oxidative stress, which is thought to contribute to the poor clinical outcome in chronic kidney disease (CKD) patients [1]. Thus, there is a need for simple and cheap biomarkers to assess oxidative stress in these patients. One such protein biomarker for oxidative stress, described in 1996 by Witko-Sarsat et al. [2] is advanced oxidation protein products (AOPP). In comparison with healthy subjects, the plasma AOPP levels have been shown to be higher in CKD patients, and furthermore, to increase with the deterioration of the renal function [2,3]. The AOPP fraction comprises several chromophores including di-tyrosine containing cross-linked components of albumin [2,4]. However, analyses of the molecular species and spectral properties of AOPP have shown also carbonyls and pentosidine, resulting partly from myeloperoxidase derived oxidative stress in hemodialysis (HD) patients, to contribute to the AOPP fraction [5,6]. Furthermore, since the AOPP concentration is about 50% [3] or even 300% [7] higher in plasma than in serum (where clotting factors have been removed), fibrinogen has been suggested as a key molecule responsible for the plasma AOPP reactivity [7].

We recently described the confounding effect of endogenous plasma compounds (mostly caused by turbidity of the triglycerides at 340 nm) rendering severe overestimation of the AOPP levels [3] using the original assay for AOPP [2] (abbreviated as oAOPP in the following). Plasma samples from uremic CKD patients are more turbid than samples from healthy individuals and the turbidity increases further if samples are thawed and re-frozen repeatedly. Also, since undissolved fat increases in the blood after a meal, postprandial plasma samples from healthy subjects may have oAOPP levels as high as those of uremic patients [3]. In addition, in our hands the oAOPP assay yields values that are only weakly correlated to other surrogate markers for oxidative stress [3]. All these findings show that the oAOPP assay yields values that do not accurately reflect oxidative stress. This was also a pronounced formulated concern of the founders of the oAOPP assay, who pointed out that dyslipidemia and haemolysis could interfere with the AOPP measurements [6].

The following facts encouraged us to attempt to modify the oAOPP assay; (a), the oAOPP levels are increased in CKD in comparison to healthy controls at similar triglyceride levels [3] and (b), centrifuga-

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doi:10.1016/j.cca.2008.03.029

Table 1
Effect of HDL-cholesterol precipitation reagent (n=218) and ether extraction (n=60) respectively on plasma concentrations (and % remaining concentrations) of AOPP, triglycerides, cholesterol, total protein and albumin in plasma samples of HD patients

	Basal levels obtained with the original AOPP assay	Basal levels obtained with the original AOPP assay[a]	HDL-cholesterol precipitation (% remaining)	HDL-cholesterol precipitation (% remaining)[a]	Ether extraction (% remaining)[a]
	n=218	n=60	n=218	n=60	n=60
AOPP (μmoL/L)	273±157	288±193	138±30 (51±19%)	155±39 (54±20%)	131±28 (45±15%)
Triglycerides (mmoL/L)	1.86±1.07	1.92±1.25	0.21±0.12 (13±7%)	0.24±0.17 (13±14%)	0.14±0.10 (7±8%)
Cholesterol (mmoL/L)	4.20±0.92	4.37±0.90	0.95±0.36 (23±39%)	0.95±0.33 (22±37%)	0.02±0.04 (1±4%)
Total protein (g/L)	67.6±5.7	66.9±5.8	65.8±5.9 (97±4%)	64.4±5.6 (96±3%)	66.5±5.4 (99±7%)
Albumin (g/L)	36.0±3.4	34.9±3.4	36.0±3.5 (100±3%)	34.9±3.4 (100±0%)	36.8±3.6 (105±2%)

Note that neither HDL-cholesterol precipitation nor ether extraction affected the sample protein levels significantly.
[a] Among the 218 patients, samples from 60 patients were randomly selected to undergo ether extraction. For comparison the results of these 60 patient samples are presented for the three groups.

tion [3] and other delipidation procedures [2] which clarify plasma from turbidity, result in decreased oAOPP values. Thus, based on the concerns raised in our recent study [3], the aims of the present work was to refine this assay, and to apply the modified methodology (abbreviated as mAOPP in the following) on stored samples from HD patients. The mAOPP assay proposed contains a precipitation procedure, which, by the addition of dextran sulphate and magnesium ions reduces the lipoproteins (VLDL and LDL) in the plasma to a minimum. In addition, we investigated if the poor associations between the oAOPP assay and other surrogate markers for oxidative stress improve using the mAOPP assay.

2. Methods

2.1. Study populations and samples

Studies were carried out in 218 prevalent HD patients (101 females); aged 63±14 years with a dialysis vintage time of 53 (1–378) months. Samples from thirteen healthy controls; aged 44±10 years were analyzed to show the potential difference against the HD group in remaining AOPP levels after lipid precipitation. Frozen, but not previously thawed plasma samples from HD patients were used throughout this study. Venous blood samples were collected under non-fasting conditions in appropriate tubes for the generation of plasma and serum. All HD patients were sampled before the dialysis sessions. Non-fasting blood containing various triglyceride levels was used in this study as our aim was to study potential interferences by the lipids. All blood samples were centrifuged immediately at 2300 ×g for 15 min at 4 °C and stored at −70 °C pending analyses. The study protocol was approved by the Ethics Committee of Karolinska University Hospital Huddinge and informed consent was obtained from each patient and control subject.

2.2. The modified AOPP (mAOPP) assay

The mAOPP assay included, in addition to the oAOPP methodology [2], a sample preparation procedure to precipitate lipoproteins (VLDL and LDL) in the plasma (Konelab™ HDL-cholesterol precipitating reagent, Thermo Electron Corporation, Vantaa, Finland). This reagent is normally used as a preparation step before determination of HDL-cholesterol on Konelab analyzers. Fifty μL of reconstituted precipitating reagent (dextran sulphate and magnesium ions) was mixed with 500 μL of EDTA plasma, centrifuged at 1000 ×g for 20 min, upon which the supernatant was carefully removed. mAOPP were immediately measured in the supernatant at 340 nm on a microplate spectrophotometer (SPECTRAmax™ 250, Molecular Devices Corporation, CA, USA) under acidic conditions and expressed as chloramine-T (Sigma Chemical Company, St. Louis, MO, USA) equivalents [2]. All control and HD samples were also analyzed with the oAOPP assay and the results obtained from the two assays were compared.

An alternative, but more laborious, extraction method using butanol and di-isopropylether in a 40:60 (v/v) ratio [8] was tested in 60 HD samples for comparison and in order to validate the dextran sulphate precipitation method. In the alternative method, 0.5 mL plasma was mixed with 1 mL extraction reagent in a test tube, vigorously shook and centrifuged at 400 ×g. The plasma proteins remained resolved in the aqueous phase following the lipid extraction procedure.

2.3. Other blood analyses

The inflammation biomarkers interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured on an Immulite® Automatic Analyzer (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG, a biomarker of oxidative DNA damage) was analyzed with a competitive high sensitivity Elisa (JAICA, Fukuroi City, Japan). Pentosidine, an advance glycation end product (AGE) biomarker, was measured with HPLC [9]. HDL-cholesterol, white blood cells, fibrinogen and high sensitivity C-reactive protein (hsCRP, analyzed with nephelometry) were measured by routine procedures. Triglycerides, S-albumin (bromcresol green), cholesterol and total protein were analyzed on a Konelab 20XT analyzer from Thermo Electron Corporation, (Vantaa, Finland).

2.4. Statistical analyses

Data are given as mean±SD. The Student's t-test or Wilcoxon test was used for paired comparisons, as appropriate. The association between variables was evaluated by the linear regression and expressed as r^2 and significance. Statistical significance was accepted at a p-value<0.05.

3. Results

The mAOPP assay consists of a simple precipitation procedure, which was carried out at a negligible cost (0.25 €/sample) and time

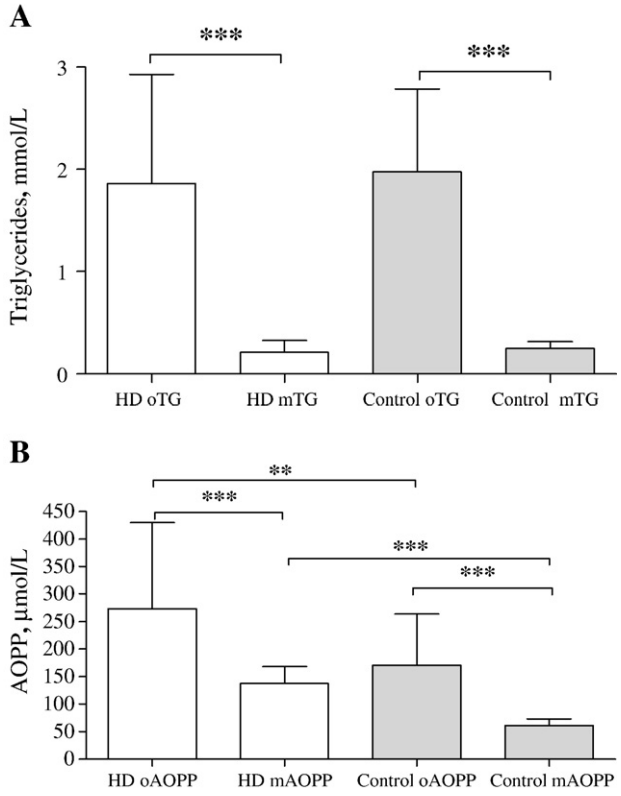

Fig. 1. Triglycerides (A) and AOPP concentrations (B) in samples from 218 HD patients and 13 controls analyzed with the original (oAOPP) and the modified AOPP (mAOPP) assays. The only difference between the two assays was that the modified assay included a simple precipitation step for the lipids (Konelab™ HDL-cholesterol precipitating reagent). The statistical significances are marked as **; p<0.01; ***; p<0.001. Significant differences were shown in the figures.