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Nitrated apolipoprotein AI/apolipoprotein AI ratio is increased in diabetic patients with coronary artery disease

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ABSTRACT

Aims/hypothesis: Recent studies have suggested that determination of HDL function may be more informative than its concentration in predicting its protective role in coronary artery disease (CAD). Apolipoprotein AI (apoAI), the major protein of HDL, is nitrosylated *in vivo* to nitrated apoAI (NT-apoAI) that might cause dysfunction. We hypothesized that NT-apoAI/apoAI ratio might be associated with diabetes mellitus (DM) in CAD patients.
Methods: We measured plasma NT-apoAI and apoAI levels in 777 patients with coronary artery disease (CAD) by ELISA. Further, we measured plasma cholesterol efflux potential in subjects with similar apoAI but different NT-apoAI levels.
Results: We found that median NT-apoAI/apoAI ratio was significantly higher in diabetes mellitus (DM) (n = 327) versus non-diabetic patients (n = 450). Further analysis indicated that DM, thiobarbituric acid-reactive substances and C-reactive protein levels were independent predictors of higher NT-apoAI/apoAI ratio. There was negative correlation between NT-apoAI/apoAI and use of anti-platelet and lipid lowering drugs. The cholesterol efflux capacity of plasma from 67 individuals with differing NT-apoAI but similar apoAI levels from macrophages *in vitro* was negatively correlated with NT-apoAI/apoAI ratio.
Conclusions: Higher NT-apoAI/apoAI ratio is significantly associated with DM in this relatively large German cohort with CAD and may contribute to associated complications by reducing cholesterol efflux capacity.

1. Introduction

Diabetes mellitus (DM), a well known risk factor of atherosclerosis, is responsible for a three to four fold increase in cardiovascular events [1]. It is known that hyperglycemia stimulates the production of advanced glycosylated end products, activates protein kinase C, and enhances the polyol pathway leading to increased superoxide anion formation [2,3]. Superoxide anion interacts with nitric oxide to form a strong oxidant peroxynitrite, which reacts with tyrosines in various proteins to generate nitrotyrosine (NTyr) residues. Therefore, levels of total plasma NTyr provide a composite index of reactive nitrogen species. Another

Abbreviations: ABCA1, ATP-binding cassette family A protein 1; ACS, acute coronary syndrome; ANOVA, analysis of variance; apoAI, apolipoprotein AI; BMI, body mass index; BSA, bovine serum albumin; CAD, coronary artery disease; CRP, C-reactive protein; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; LSD, least significant difference; NTyr, nitrotyrosine; NT-apoAI, nitrated apoAI; NYHA, New York Heart Association; PAD, peripheral artery disease; PBS, phosphate buffer saline; PBST, phosphate buffered saline containing Tween; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TG, triglyceride; TIA, transient ischemic attack.

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pathway of the NTyr production involves reaction of nitrogen dioxide ($\cdot NO_2$) with tyrosine residues [4]. $\cdot NO_2$ is generated by myeloperoxidase from hydrogen peroxide (H_2O_2). Several small population studies have shown that NTyr levels are increased in DM [5–10] and coronary artery disease (CAD) [11,12].

Of various proteins present in plasma, apolipoprotein AI (apoAI) has been shown to be a preferred substrate for modification by reactive nitrogen species in human artery wall [13]. Circulating nitrated apoAI (NT-apoAI) levels are 7-70-fold higher in comparison with other nitrated proteins [13,14]. In the apoAI molecule, tyrosine residues 166 and 192 have been shown to be the predominant sites of nitration by myeloperoxidase [15–17]. *In vitro* nitration of apoAI impairs lipid binding and ATP-binding cassette family A protein 1 (ABCA1)-dependent cholesterol efflux functions [16]. However, physiologic effects of *in vivo* NT-apoAI are unknown.

In a small case–control study, the circulating NT-apoAI/apoAI ratio was significantly increased in diabetic patients compared with controls [18]. In addition, higher levels of NT-apoAI were found in patients with cardiovascular disease in both the circulation and in atheroma [13,14,16]. Recently, Vazquez et al. reported a significantly higher level of circulating NT-apoAI in obese women [19]. Although there appears to be a tendency towards higher plasma NT-apoAI levels in diabetes and CAD subjects, the studies were small and used gas chromatography-mass spectrometry methods that are not suitable for larger cohort studies. Previously, we developed a novel [20] enzyme-linked immunosorbent assay (ELISA), which was confirmed by both Western blot analysis [20] and mass spectrometry [19]. This sensitive and specific ELISA method is suitable to quantify human NT-apoAI levels in a large population. In the present study, we hypothesized that NT-apoAI would be increased in diabetic patients with CAD.

2. Research design and methods

2.1. Study population

A total of 777 subjects with CAD admitted to the Department of Medicine III (Angiology and Cardiology) and the Department of Heart and Thoracic Surgery, University Clinic Halle, Germany between January, 2008 and December, 2010 were prospectively studied. As expected, very few normal subjects were admitted during this period precluding comparison of different parameters between normal and CAD patients. The data presented include a retrospective analysis of measured NT-apoAI and apoAI levels. CAD was diagnosed as the presence of a luminal diameter stenosis ≥50% in at least one major coronary artery (left anterior descending, left circumflex or right coronary artery or their major branches) by angiography or a history of myocardial infarction, among which 380 patients had stable angina pectoris and 397 had acute coronary syndrome (ACS). Multi-vessel CAD was defined as a disease stage in which at least two of the major coronary arteries is involved with atherosclerosis of significant severity. Within this cohort, 327 patients had DM and 450 patients had no DM. DM was diagnosed at the time of admission by past clinical history of a fasting blood glucose level >7.0 mmoL/L (>125 mg/dL) and/or the two-hour value in the 75 g oral glucose tolerance test (OGTT) > 11.1 mmoL/L (200 mg/dL) or use of hypoglycemic medications. HbA1c and insulin levels were not determined. At the time of admission, the history of current smoking, hypertension, dyslipidemia, peripheral artery disease (PAD), previous stroke or transient ischemic attack (TIA), New York heart association (NYHA) functional classification, renal failure, and use of anti-platelet drugs and lipid lowering drugs was collected. Hypertension was defined as patients receiving antihypertensive treatment or having a previous diagnosis of hypertension (blood pressure ≥ 140/90 mmHg). The study was approved by the ethics committee of the medical faculty of the Martin Luther-University Halle-Wittenberg.

2.2. Laboratory evaluation

Subjects were instructed to fast for at least eight hours prior to blood sampling. Serum samples were collected and stored at −80 °C prior to use. Laboratory tests were performed at the time of hospital admission for triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and C-reactive protein (CRP) as described before [21]. For the evaluation of renal function, estimated glomerular filtration rate (eGFR) was calculated by the abbreviated Modification of Diet in Renal Disease (MDRD) [22] Study equation: 186 × (Creatinine/88.4)−1.154 × (Age)−0.203 × (0.742 if female) × (1.210 if black). Plasma glucose levels were measured (Autokit glucose, Wako, #439-90901) in 71% (550 subjects) of the population, among which 249 patients had DM and 308 patients had no DM.

2.3. Measurement of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation in human plasma was quantified by measuring the formation of TBARS using a kit. Plasma (10 μL) was mixed with 10 μL SDS solution. Then 125 μL TBARS solution (0.53 g trichloroacetic acid dissolved in 100 ml of sodium hydroxide and 100 ml of sodium dodecyl sulfate) was added to the sample. After incubation at 95 °C for 60 min and keeping at 4 °C for 15 min, the sample was centrifuged at 3000 rpm for 15 min. The amount of TBARS produced was measured at 532 nm. Malondialdehyde was used to construct a standard curve in parallel.

2.4. Standards used for the quantification of NT-apoAI and apoAI

Purified human HDL (MyBioSource, # MBS173147, total protein 40 mg/ml, standard curve range 1–12 ng/ml) was used as standard for the determination of plasma concentrations of apoAI. For NT-apoAI standard, purified human apoAI (Biomedical Technologies, # BT-927) was nitrated with sodium nitrite [23,24]. Briefly, purified apoAI (~1 mg/ml) was adjusted to pH 3.5 and mixed with 200 mM sodium nitrite and then incubated at 37 °C for 24 h under low agitation. Samples were dialyzed against phosphate buffer saline (PBS) for 24 h at 4 °C, and protein concentration was determined by the Coomassie Reagent (Thermo Scientific, #23236) and bovine serum albumin (BSA) as a standard [25]. The amounts of nitrated tyrosines in the modified apoAI were determined using a commercially available NTyr ELISA kit based on nitrated albumin (Cell Biolabs, # STA-305) as described by the manufacturer. The nitrated apoAI (0.7 mg/ml protein) contained 2000 nM NT-apoAI giving a concentration of 80 mmol of nitrated tyrosine per mol of apoAI.

2.5. ELISA procedure for the measurement of NT-apoAI and apoAI

Serum NT-apoAI was measured by a sandwich ELISA as described before [20]. Briefly, microtiter plates were coated with capturing monoclonal anti-NTyr antibodies (1 μg/mL, Upstate, # 05-233, clone 1A6) in PBS overnight at 4 °C. The non-specific sites were blocked by incubating the wells with 200 μL of phosphate buffered saline containing 0.05% Tween (PBST) and 3% BSA for 1 h at room temperature. After washing three times with PBST, patient samples (1:25,000 dilution in PBST containing 3% BSA) or standard controls (10–100 ng/mL, nitrated human apoAI) were added to each well and incubated for 1 h at 37 °C, enabling the binding of all the nitrated proteins to immobilized monoclonal antibodies. The