Assessment of microvascular reactivity and oxidative stress in hypertensive adolescents and hemodialysis patients

Ph.D. Thesis

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Szeged, Hungary
2010

LIST OF ABBREVIATIONS

ACH	Acetylcholine
BMI	Body mass index
BP	Blood pressure
CKD	Chronic kidney disease
E-CAT	Erythrocyte catalase
E-MDA	Erythrocyte malondialdehyde
ESA	Erythropoiesis-stimulating agent
E-SOD	Erythrocyte superoxide dismutase
ESRD	End-stage renal disease
FMD	Flow-mediated dilation
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GSSG/GSH	Ratio oxidized/reduced glutathione
Hb	Hemoglobin
HD	Hemodialysis; hemodialyzed
HDL	High-density lipoprotein
LDF	Laser Doppler flowmetry
LDI	Laser Doppler imaging
LDL	Low-density lipoprotein
PORH	Postocclusive reactive hyperemia
PWV	Pulse wave velocity

1. INTRODUCTION

1.1. Oxidative stress in pathological states

Reactive oxygen and nitrogen species are products of the normal cellular metabolism and play important roles in various physiological processes. Under normal conditions, the cell maintains a balance between the prooxidants and antioxidants, a state referred to as “redox balance” or “redox homeostasis”. Defense mechanisms against elevated concentrations of reactive species involve enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and the peroxiredoxin–thioredoxin–thioredoxin reductase system; and nonenzymatic scavenging antioxidants, represented by reduced glutathione (GSH), alfa-tocopherol (vitamin E), urate and other antioxidants.

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage to nucleic acids, lipids and proteins. Oxidative modifications of these biomolecules can be detected in cardiovascular (e.g. hypertension, obesity and atherosclerosis), renal (chronic kidney disease (CKD) and end-stage renal disease (ESRD)) and pulmonary diseases (e.g. asthma and cystic fibrosis), and also in cancer and diabetes. These modified molecules can serve as representative biomarkers of the oxidative/nitrosative damage involved in the pathophysiology of the diseases.

The definition of juvenile hypertension is based on the normative distribution of blood pressure (BP) in healthy children, as a systolic and/or diastolic BP $\geq 95^{th}$ percentile for age, gender and height (on at least three separate occasions). The prevalence of hypertension in childhood and adolescence (about 3% in 2006) is showing an increasing trend, which is in part attributable to the high prevalence of obesity. BP and weight show tracking from childhood into adulthood, which further emphasizes the importance of juvenile hypertension and overweight.

The prevalence of overweight in adolescents varies from under 5% to more than 20%, the exact number being dependent on geographical and ethnical factors, and the definition of obesity itself. The body mass index (BMI) is the parameter most widely

used to assess childhood overweight, even if it is only an indirect measure of adiposity. Due to the fact that the BMI in children varies greatly with age and sex, percentiles, or age and sex-specific cut-off points are used to define childhood overweight and obesity.

The interrelation between overweight and hypertension is complex, involving (among others) activation of the sympathetic and renin-angiotensin-aldosterone systems, and various metabolic and vascular changes (e.g. insulin resistance, decreased nitric oxide availability and renal sodium excretion, and increased levels of plasma lipids and endothelin-1). These effects have been suggested to be mediated, in part, by hyperleptinemia and leptin resistance in obesity. We earlier demonstrated that oxidative stress is an important feature in juvenile hypertension with or without obesity, and that a short-term antihypertensive treatment can reveal differences in markers of oxidative stress and endothelial dysfunction between lean or obese hypertensive adolescents. These finding suggest that the pathomechanisms of juvenile hypertension associated with normal body weight or obesity may differ.

The incidences of CKD and ESRD show increasing trends, in part due to the ever more frequent prevalence of their underlying diseases, in particular diabetes. Besides classical cardiovascular risk factors (e.g. diabetes, hypertension and dyslipidemia), uremia-associated factors such as a chronic volume expansion, inflammation, oxidative stress and anemia also play roles. Uremia itself is regarded as a prooxidant state, and the oxidative stress becomes more severe together with the severity of CKD. Despite beneficial effects due to the removal of uremic toxins and excess fluid, hemodialysis (HD) sessions may themselves induce repetitive bouts of oxidative stress. Anemia is a common feature of CKD and ESRD, and is associated with elevated levels of morbidity and mortality in HD patients. The fundamental treatment choices in anemia correction are the administration of erythropoiesis-stimulating agents (ESAs), iron and adjuvant vitamins, and in HD patients, optimization of the dialysis process. Despite a transient increase in the levels of oxidative markers after the initiation of ESA therapy (preventable with simultaneous vitamin E administration), long-term ESA treatment is thought to have antioxidant effects, though the mechanisms have not been well established.

1.2. Assessment of the vascular system in pathological states

Among the various techniques developed for the assessment of large vessels, the *measurement of arterial stiffness* is being increasingly used in the clinical setting. The carotid-femoral pulse wave velocity (PWV; considered the gold-standard measurement of arterial stiffness) is elevated in adult patients with obesity and uncomplicated essential hypertension, and in those on HD, and was shown to be of independent predictive value for all-cause mortality, cardiovascular morbidity, coronary events and stroke. Forearm *venous occlusion plethysmography* is often used in combination with complete occlusion and subsequent release of the arm (postocclusive reactive hyperemia (PORH) test) or brachial artery catheterization. The impaired endothelial function of the brachial artery was confirmed in both hypertensive adults and HD patients in earlier studies in which this technique was used. In the assessment of *brachial artery flow-mediated dilation (FMD)*, the changes in the arterial diameter after the PORH test, or due to a sublingual dose of nitroglycerin, are monitored by means of vascular ultrasonography. FMD has been reported to be impaired in obese children and in patients on HD. A decreased brachial artery FMD in adult hypertension has been suggested to have a prognostic role in identifying patients at higher risk of nonfatal or fatal cardiovascular events.

The definition of “microcirculation” is based on the vessel physiology rather than diameter or structure. All vessels that respond to increasing pressure by a myogenic reduction in lumen diameter are considered part of the microcirculation. Such a definition includes the smallest arteries and arterioles in the microvasculature in addition to capillaries and venules.

Laser Doppler flowmetry (LDF) and *laser Doppler imaging (LDI)* are noninvasive means of assessment of the microvascular function. This technique is based on the scattering and frequency-shift of monochromatic laser light due to its interaction with moving blood cells. Laser Doppler methods measure the microvascular blood perfusion (termed the flux), which is the product of the velocity and concentration of the moving blood cells within the measured volume. LDI makes use of a laser beam that scans across a predetermined area, resulting in a two-dimensional map of the blood perfusion.

LDF, in turn, allows continuous monitoring of the flux at a specified point of the skin, offers higher sensitivity and has the ability to detect rapid perfusion changes. Due to the relatively large spatial and temporal variabilities of the technique, the response of the microvessels to provocation tests is assessed, instead of the basal flux. These tests include thermal challenges (local or systemic heating or cooling), iontophoretic administration of vasoactive drugs, the PORH test, or a combination of them.

The skin microvascular reactivity, as measured by means of the laser Doppler technique, has been reported to correlate with other methods applied to assess the vascular function in distinct vascular beds, including brachial artery FMD and transthoracic coronary echocardiography. The functional impairment of the microvascular system in HD affects both the endothelium-dependent and -independent vasodilations. In contrast, only the endothelium-dependent microvascular reactivity is affected in adult hypertension, whereas the endothelium-independent function is preserved. Although LDF is highly appropriate for the examination of children and adolescents (due to its simple, convenient and painless nature), no data were available regarding the functional properties of the microvasculature in juvenile essential hypertension before our studies.

1.3. Aims

1. Is there a detectable alteration in the microvascular reactivity in juvenile essential hypertension? Is the microvascular reactivity impaired or augmented? Is the endothelium-dependent or the -independent reactivity changed?
2. Is there any variance in the microvascular reactivity as a function of the BMI in adolescent hypertensives?
3. Does the level of oxidative stress correlate with the microvascular reactivity in juvenile hypertensives and HD patients?
4. Do the characteristics of the treatment with ESA (the type and the withdrawal of ESA) influence the oxidative stress in HD?

2. SUBJECTS AND METHODS

2.1. Microvascular reactivity assessment – laser Doppler flowmetry

Microvascular reactivity studies were performed in a temperature-controlled room (24 °C) between 9 a.m. and noon, the subjects being in the fasted state. They were lying in a supine position with their feet at the heart level. After a 15-min equilibration, the volar surface of the right forearm (in HD patients, the forearm contralateral to the Cimino fistula) was gently cleaned with alcohol. The microvascular perfusion of the skin was continuously measured at two sites (distance of separation: 6-8 cm) by means of a DRT4 laser Doppler flowmeter (laser wavelength: 780 nm, Moor Instruments Ltd., Axminster, UK). The optic probes (DP12-V2) fit into the heater probes (SHP2), which, in turn, fit into the iontophoresis chambers (ION1, surface area: 0.71 cm^2; all Moor Instruments Ltd., Axminster, UK). This setting allows perfusion measurements at skin sites affected by iontophoretic and local thermal provocations. The positions of the probes were chosen so as to avoid hair and injured skin. A MIC2 Iontophoresis Device (Moor) was utilized for the parallel delivery of freshly prepared 1% isotonic saline solutions of the endothelium-dependent vasodilator acetylcholine (ACH) and the endothelium-independent vasodilator sodium nitroprusside (SNP) (both Sigma-Aldrich, St. Louis, MO, USA), using separate chambers (at sites 1 and 2, respectively). The local skin temperature was standardized at 33 °C during the iontophoresis sequence, and subsequently increased to 44 °C by means of a SH02 Skin Heating Unit (Moor).

The LDF protocol consisted of a 4-min measurement of the baseline perfusion, followed by 3 consecutive, increasing iontophoretic doses of ACH and SNP (current: 20 μA, duration: 20, 40 and 80 s, respectively; yielding a total charge of 2.8 mC and a total charge density of 3.94 mC/cm^2). Between iontophoretic doses, 4-min intervals with no current administration were allowed. Following an additional 10-min interval after completion of the iontophoresis sequence, both measurement sites were gradually heated at a rate of 0.1 °C/s to reach and maintain a temperature of 44 °C for 25 min, corresponding to maximal vasodilation.

Flux values at baseline and in the plateau phase of the local heating response were evaluated by averaging 60-s intervals. Average values of 20-s intervals were used as peak perfusion responses after each iontophoretic dose. All counted perfusion values were expressed relative to the baseline values (baseline=100%). The within-subject variabilities determined at fixed skin sites of healthy young subjects were 15-25% for both iontophoretic and local thermal provocations in our laboratory.

In order to validate the LDF protocol in the clinical setting, the microvascular reactivities of 12 HD patients were assessed. In agreement with the literature, the endothelium-dependent and -independent vasodilation both proved to be markedly diminished as compared with those in healthy young subjects (data not published). The study protocol was well tolerated by both the patients and the controls, and no adverse local events (pain or red flare) or systemic effects (change in BP, heart rate or respiratory rate) were observed.

2.2. Laboratory determinations

In Study 1 (Microvascular reactivity in lean, overweight and obese hypertensive adolescents), venous blood was obtained prior to the LDF measurements. In Study 2 (Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis), we used blood samples drawn from the arterial line of the dialysis tubes either at the end of the long interdialytic period (biochemical markers) or during routine mid-week blood sampling (hematological parameters), respectively.

Whole blood levels of oxidized glutathione (GSSG) and GSH were assayed by means of a spectrophotometric enzymatic recycling method. Plasma alfa-tocopherol and erythrocyte malondialdehyde (E-MDA) were determined by means of high-performance liquid chromatography, and a variable-wavelength ultraviolet-visible detector. The levels of plasma alfa-tocopherol are reported relative to the total cholesterol plus triglyceride levels. The activities of the erythrocyte SOD (E-SOD) and CAT (E-CAT) were measured with spectrophotometric methods, and are given relative to the protein concentration of the sample (determined by means of spectrophotometry). Plasma levels of the total, high- and low-density lipoprotein (HDL and LDL) cholesterol and

triglycerides, as well as hemoglobin (Hb) levels, proportions of reticulocytes, transferrin saturation values and ferritin levels, were determined with standard laboratory methods.

2.3. Study groups and interventions

2.3.1. Microvascular reactivity in lean, overweight and obese hypertensive adolescents (Study 1)

Hypertension was defined as a 24-h systolic and/or diastolic mean BP equal to or greater than the 95th percentile for age, height and sex, measured with an oscillometric ambulatory BP monitor (Meditech ABPM-04, Budapest, Hungary). Secondary causes of hypertension (renal parenchymal, renovascular, endocrinological, cardiological or neurological) were excluded. No proteinuria (defined as >10 mg/kg/day) or any impairment in renal function (creatinine clearance <80 ml/min/1.73 m^2) was observed. Of the 33 adolescents (aged 6-19) enrolled after establishment of the diagnosis of essential hypertension in the Department of Pediatrics, University of Szeged, 10 patients had a normal BMI (lean hypertensive (LH) group), while 13 adolescents were classified as overweight hypertensive (OWH) and 10 patients as obese hypertensive (OBH) according to the age and sex-specific cut-off points of child overweight and obesity defined by the International Obesity Task Force. Nineteen healthy adolescents with no evidence of cardiovascular or renal disorders served as controls, recruited from secondary schools in Szeged. For all groups, exclusion criteria included the presence of an acute illness, smoking, and taking medication (in the previous 5 days) or beverages (on the previous day) known to affect microvascular perfusion. No signs of acute inflammation or systemic infection were revealed by means of physical examination and qualitative blood count in any of the patients or controls. Written informed consent was obtained from the patients, the controls and their parents prior to the study, which was approved by the Ethical Committee of the University of Szeged.

2.3.2. Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis (Study 2)

Twenty-one patients on chronic HD were enrolled in Study 2, dialyzed in two distinct morning shifts (11 patients: age (mean)±SD 59.3±17.7 yrs, time on HD: 3.6±2.0 yrs; and 10 patients: age 56.6±13.6 yrs, time on HD: 4.3±2.1 yrs, respectively). All patients were on 4-h bicarbonate HD, performed on a Polyflux 21L dialyzer, 3 times a week. The single-pool Kt/V was greater than 1.4, in line with the current European Best Practice Guideline on Dialysis Strategies. Heparin was used as the anticoagulant during HD. The primary diagnoses were chronic glomerulonephritis, chronic pyelonephritis or hypoplastic kidney. No patients had hypertension or diabetes as etiological factors for chronic kidney disease in these shifts. Fifteen of the patients were undergoing antihypertensive treatment with amlodipine, enalapril, metoprolol or prazosin. Additional drugs regularly used by some patients included sodium polystyrene sulfonate, sevelamer, clopidogrel, acetylsalicylic acid and famotidine. All patients received 5 mg folate once weekly. Patients with diabetes were excluded. The regular examination of the patients did not reveal any sign of acute inflammation or systemic infection throughout the study.

The patients had been receiving epoetin beta twice weekly. As part of the treatment strategy, the latter 10 patients were to be switched to darbepoetin alfa, independently from the present study. Epoetin beta therapy was withdrawn for 14 days, after which ESA administration was resumed either with epoetin beta (11 patients, dose: 5000 IU twice a week), or with darbepoetin alfa (10 patients, dose: 50 µg once weekly after the first dialysis of the week), administered in subcutaneous bolus injections at the end of the dialysis. If the transferrin saturation of a patient fell below 30%, 62.5 mg iron gluconate was given intravenously during each of five consecutive dialysis sessions. Written informed consent was obtained from the patients prior to their entering the study, approved by the Ethical Committee of the University of Szeged.

2.4. Statistical analysis

The results are reported as medians (ranges) and means±SD for Studies 1 and 2, respectively. Statistical analyses were performed with GraphPad Prism 4.00 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons included the nonparametric Kruskal-Wallis test, followed by Dunn's multiple comparison test (Study 1), or repeated-measures two-way analysis of variance, followed by Bonferroni's *post hoc* test (Study 2). For both studies, a p value <0.05 was considered significant.

3. RESULTS

3.1. Microvascular reactivity in lean, overweight and obese hypertensive adolescents (Study 1)

The plasma levels of HDL cholesterol were significantly elevated in the LH group, and significantly decreased in the OBH patients (both $p<0.05$ *vs* the controls). The plasma triglyceride levels were higher in the OWH and OBH patients as compared with the controls ($p<0.05$ and $p<0.01$, respectively). The plasma HDL cholesterol and triglyceride levels in the OBH group were also higher than those in the LH subjects ($p<0.01$ and $p<0.05$, respectively). The mean age, diastolic BP and plasma levels of total and LDL cholesterol were similar in the study groups.

The whole blood ratios oxidized/reduced glutathione (GSSG/GSH) were significantly elevated in all the patient groups (all $p<0.001$ *vs* the controls). Also, the ratios GSSG/GSH in the OBH group were higher than those in the LH subjects ($p<0.05$). The activities of E-CAT were increased in the OWH group as compared with the controls ($p<0.05$). The levels of plasma alfa-tocopherol (relative to the total cholesterol plus triglyceride levels) and E-MDA, and the activities of E-SOD were similar in the study groups.

As concerns laser Doppler flowmetry measurements, the perfusion increments after iontophoresis of the endothelium-dependent vasodilator ACH did not differ significantly in the patient groups and the controls. In contrast, the second iontophoretic dose of the endothelium-independent vasodilator SNP increased the microvascular blood flux to a significantly smaller extent in the LH group than in the controls (482.5%

(157.0-842.0%) *vs* 847.0% (432.0-1708.0%); $p<0.05$). After the third SNP dose, similar differences were revealed in the LH and the OBH groups as compared with the controls (797.5% (270.0-1100.0%) *vs* 1195.0% (674.0-1774.0%) and 744.0% (390.0-1207.0%) *vs* 1195.0% (674.0-1774.0%), respectively; both $p<0.05$).

In response to local heating to 44 °C, the increase in the microvascular perfusion attained a plateau at similar relative flux values at site 1 in all groups. At site 2, significantly smaller flux increments were detected in the LH and the OBH groups than in the controls (1257.0% (585.0-1649.0%) *vs* 1870.0% (1029.0-2970.0%) and 1188.0% (693.0-1815.0%) *vs* 1870.0% (1029.0-2970.0%), respectively; both $p<0.05$). The study protocol was well tolerated by all subjects and no adverse local or systemic effects were observed.

3.2. Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis (Study 2)

The Hb levels were decreased at week 6 in both groups ($p<0.05$ *vs* the baseline), and had returned to the initial values by week 14. The proportion of reticulocytes exhibited a decrease at week 2 and a subsequent elevation at weeks 6 and 10 (all $p<0.05$ *vs* the baseline). No differences were observed between the groups in either parameter. The transferrin saturation values and the levels of ferritin were not changed in either group.

The ratios GSSG/GSH were significantly increased at weeks 2 and 6 in both groups (all $p<0.001$ *vs* the baseline), but had returned to the initial levels by week 14. Similar tendencies were found in the levels of GSSG. The GSH concentrations were increased at week 14 in the two groups ($p<0.001$ *vs* the baseline). The E-MDA levels exhibited an elevation at week 6 ($p<0.01$ *vs* the baseline) and a subsequent return to the initial levels by week 14 in both groups. As compared with the baseline values, the activities of E-SOD were reduced significantly at week 6 in both study groups ($p<0.001$), and had returned to the baseline by week 14. The activities of E-CAT were significantly increased at week 14 in both groups ($p<0.001$ *vs* the baseline).

4. CONCLUSIONS AND ORIGINAL FINDINGS

1. The endothelium-dependent microvascular reactivity is not significantly attenuated in hypertensive adolescents being lean, overweight or obese. This finding suggests that, in the pathogenesis of juvenile essential hypertension, an impairment of the endothelium-dependent microvascular reactivity is more likely to follow, rather than precede the elevation of the BP.
2. The endothelium-independent vasodilation is significantly impaired in the LH and OBH groups, but not in the OWH patients, as compared with the controls.
3. The microvascular reactivities are not related to differences in the levels of oxidative markers, even if the presence of an increased oxidative stress is confirmed in all the hypertensive groups.
4. An increased oxidative stress is revealed by significantly elevated ratios GSSG/GSH directly after the withdrawal of epoetin beta treatment in HD patients.
5. The levels of GSSG/GSH, GSSG and E-MDA are significantly increased and the activities of E-SOD are significantly decreased four weeks after darbepoetin alfa or epoetin beta therapy resumption as compared with the baseline.
6. The levels of GSSG/GSH, GSSG and E-MDA return to the baseline values by the end of the 12-week follow-up, parallelled with increased GSH and E-CAT activity levels.
7. The opposite trend in the levels of Hb and oxidative markers, and similarities in the time courses and magnitudes of the oxidative alterations during the treatment with different ESAs suggest that the observed changes may primarily be caused by factors associated with the correction of anemia, rather than the direct effect of ESA. This emerging hypothesis is yet to be confirmed.

5. ACKNOWLEDGMENTS ARE DUE TO

my wife, Dr. Ildikó Farkas, for always being there. I thank her for her love, friendship, sincerity, inspiration, patience and continuous support within scientific life and beyond,

my parents and relatives, for their holding out and for the comforting atmosphere provided,

my friends and colleagues, for their friendship and scientific talks,

Ilona Szécsi and Ágota Fábiánné Nagy, for their excellent laboratory work,

Dr. Eszter Hódi and Dr. Adrienn Máté, for their contributions in the microvascular reactivity measurements and for the useful talks,

Dr. Ilona Szöllősiné Varga and Dr. Zsuzsanna Hracskó, for their cooperation in the antioxidant enzyme measurements and their advice,

Dr. Katalin Farkas (St. Imre Hospital, Budapest, Hungary) and Peter Jady (Moor Instruments Ltd., Axminster, UK) for providing me deep insight into the laser Doppler technique,

Dr. Eszter Karg and Dr. Gyula Wittmann, for their scientific encouragement, knowledge and exemplary attitude,

Prof. Dr. Ilona Németh, for being my first supervisor and for her encouragement,

Prof. Dr. Sándor Túri, for accepting me as a Ph.D. student in the Department of Pediatrics, for the constant scientific consultation and for ensuring all the conditions needed for the experiments,

and to all participating patients, controls and their parents, without whom these advances could not have been achieved.

This work was supported by Hungarian National Scientific Research Grant OTKA K67895 and by a Research&Development Grant of the Hungarian Society of Nephrology.

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