Diagnostic Biomarkers of Essential Arterial Hypertension

The Value of Prostacyclin, Nitric Oxide, Oxidized-LDL, and Peroxide Measurements

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Summary

Endothelial function is impaired in hypertensive patients. Decreased nitric oxide and prostacyclin production as well as increased oxidative stress are involved in this abnormality.

The aim of the present study was to evaluate whether biomarkers of endothelial dysfunction and oxidative stress have diagnostic value in patients with essential hypertension.

We measured nitric oxide, prostacyclin, and oxidized-LDL levels and assessed oxidative status in 62 patients with diagnosed essential arterial hypertension and 45 healthy controls.

In the hypertensive group, among measured parameters, the median prostacyclin level was significantly lower, when compared to healthy controls (125.57 pg/mL, 25%; 75% quartile range: 84.99; 275.93 and 462.9 pg/mL, 25%; 75% quartile range: 107.69; 849.3, respectively, P = 0.009). The largest area under the ROC curve was found for prostacyclin; 0.647 (95% C.I. 0.549 to 0.737). In the analysis of logistic regression, the prostacyclin and oxidized-LDL cut-off values were associated with a 4.9 higher significant risk of hypertension (O.R. 4.91 and 4.99, respectively; P = 0.0008 and P = 0.00065, respectively). Oxidized-LDL, a biomarker of endothelial damage, was the only one that had a significant negative correlation with protective prostacyclin in hypertensive patients (r = -0.29, P = 0.02).

Of all the biomarkers prostacyclin and oxidized-LDL had the best diagnostic value for patients with hypertension. (Int Heart J 2009; 50: 341-351)

Key words: Essential arterial hypertension, Nitric oxide, Oxidative stress, Oxidized-LDL, Total peroxide concentration, Prostacyclin

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ARTERIAL hypertension is directly implicated in the pathophysiology of various cardiovascular disease states and is leading to an excess of both morbidity and mortality. The molecular basis of essential arterial hypertension (HTN) is multifactorial, complex, and poorly understood. Recent interest has been directed toward investigating the purported role of endothelial dysfunction and oxidative stress, which act as important regulators of vascular homeostasis. Vascular endothelial cell dysfunction leads to the reduction of endothelium-derived relaxing factors, of which nitric oxide (NO) seems to play the most important role. However, at this point of the pathophysiology of HTN, less well known is the role of prostacyclin (PGI2). Increased production of contracting factors, like oxygen-derived free radicals and oxidized low density lipoprotein (ox-LDL), a marker of oxidative stress, could play an important role in the development and consolidation of HTN. Determination of the balance between the formation and release of these substances is important for understanding their role in patients with HTN. In this article, we summarize the current understanding of the role of endothelium-derived relaxing factors and discuss the possibility that endothelial dysfunction may play a primary role in the pathogenesis of essential arterial hypertension. We assessed the diagnostic values of nitric oxide, prostacyclin, oxidized-LDL, and oxidative status in patients with HTN.

**Methods**

**Selection of the study population:** We selected 62 diagnosed non-smoking essential hypertensives among those referred to our hypertension outpatient clinic and compared them with 45 normotensive controls. Blood pressure was measured in an ambulatory condition using a 24-hour ambulatory blood pressure measurement device (ABPM, Tracker Reynolds NIBP2, Reynolds Medical, Hertford, UK). Cuffs of an appropriate size were used on the nondominant arm with automatic readings provided at 10 minute intervals during the day (from 6.00 a.m. to 10.00 p.m.) and every 20 minutes during the night (from 10.20 p.m. to 05.40 a.m.). Automatic deflation of the equipment was no more than 2 mmHg per second. All patients were instructed to engage in normal activities, refrain from strenuous exercise, and keep the arm extended at the time of cuff inflations. Only recordings of more than 85% of valid values were analyzed. Based on recent recommendations, HTN was diagnosed when the median 24-hour value of systolic blood pressure (SBP) was 125-130 mmHg and/or the median value of diastolic blood pressure (DBP) exceeded 80 mmHg (guidelines). Patients with a known secondary cause of HTN, any history of symptoms of coronary artery disease, diagnosed diabetes mellitus, renal dysfunction, or symptoms of heart failure (HF) were excluded. All patients were treated according to current guide-
The study design complies with the Helsinki Declaration of 1975 as revised in 1996 and it was approved by the local institutional committee on human research (Institutional Review Board-Local Bioethics Committee of Bialystok Medical University). Informed consent was obtained from all participants.

**Blood sampling and biochemical measurements:** Venous blood samples were obtained between 8.00 a.m. and 10.00 a.m. from fasting patients and healthy non-smokers. After 20 minutes venous blood samples for autoantibody IgG against oxidatively modified low-density lipoprotein and nitric oxide assays were collected into tubes with a clotting activation system, and for peroxides and 6-keto-PGF$_{1α}$ assays into tubes containing EDTA. All samples were centrifuged within 2 hours after collection and stored at -80°C until assayed.

Plasma levels of 6-keto-PGF$_{1α}$ - a PGI2 metabolite - were assayed using enzyme-linked immunosorbent assay kits (6-keto Prostaglandin F$_{1α}$ EIA Kits, Cayman Chemical Company, Ann Arbor, MI). The intra-assay coefficient of variation (CV %) for 6-keto-PGF$_{1α}$ determinations claimed by the manufacturer of the kit is 10% at a prostaglandin mean concentration of 100 pg/mL.

Serum levels of nitric oxide were measured using colorimetric Total NO/Nitrite/Nitrate assay kits (R&D Systems, Abingdon, England) according to the manufacturer’s instructions. This indirect method was based on the measurement of the more stable NO metabolites, nitrite (NO$_{2}^{-}$) and nitrate (NO$_{3}^{-}$), in serum. The assay determines nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess reaction. The intra-assay CV % is reported by the manufacturer of the assay kits to be 2.5% at a nitric oxide mean concentration of 30μmol/L (SD = 0.76 U/L).

Serum levels of IgG antibodies against oxidatively modified low-density lipoprotein were determined using enzyme-linked immunosorbent assay (Human OLAB IgG kit, Biomedica, Wien, Austria) according to manufacturer’s instructions. The intra-assay CV % is reported by the kit manufacturer to be 4.3% at an hs-CRP mean concentration of 324 U/L (SD = 14 U/L).

Plasma concentrations of peroxides were measured using a colorimetric assay kit (Oxystat, Biomedica, Wien, Austria) according to the manufacturer’s instructions. The results show a direct correlation between free radicals and circulating biological peroxides and thus allow the characterization of the oxidative status in biological samples. The peroxide concentration is determined by reaction of the biological peroxides with peroxidase and subsequent color-reaction using TMB (tetramethylobenzidine) as a substrate. After addition of a stop solution, the coloured liquid is measured photometrically at 450 nm. The manufacturer states the intra-assay coefficient of variation of the assay kit is 3.1%.
at a peroxide mean concentration of 221 $\mu$mol/L (SD = 6.9 $\mu$mol/L).

Diagnostic sensitivity, specificity, as well as positive and negative predictive values (PPV, NPV) of cut-off points characterizing patients with or without hypertension were computed. The receiver operating characteristic (ROC) curves were constructed. The area under the curve (AUC), a measure of the diagnostic efficiency, was also computed. In the ROC report, the cut-off values for measured parameters (NO, metabolite of PGI2, ox-LDL, oxidative stress) corresponding to the highest accuracy (minimal false-negative and false-positive results) were indicated.

Statistical analysis: Results are expressed as the median with 25% to 75% interquartile ranges (continuous variables) or as proportions (categorical variables). Associations between continuous variables were examined using the Mann-Whitney $U$ test and associations between categorical variables using the $\chi^2$ test. Spearman correlation was used to evaluate the relationships among parameters in the groups. Logistic regression was performed to estimate the odds ratios (OR) of the presence of hypertension for the cut-off values with optimal sensitivity and specificity, driven from the ROC analysis. All analyses were carried out using Statistica 6.0 (StatSoft, Tulsa, OK, USA) and MedCalc 8.0 (MedCalc Software, Mariakerke, Belgium). A $P < 0.05$ was considered statistically significant.

Results

Patient backgrounds and concomitant diseases: Overall, 107 subjects were studied; 62 patients (median age, 53 years, 25%; 75% quartile range: 44; 64) with ambulatory diagnosed HTN and 45 healthy controls (median age, 30 years, 25%; 75% quartile range: 22; 34). There were 35 (56%) males and 27 (44%) females in the patient group, and 11 males (24%) and 34 females (76%) in the control group. Only patients with primary (essential) arterial hypertension were assessed; patients with any secondary cause of the disease were excluded. Therefore, the exclusion criteria were renal parenchymal disease, renovascular

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HTN patients ($n = 62$)</th>
<th>Control group ($n = 45$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>25%-75% quartile range</td>
<td>Median</td>
</tr>
<tr>
<td>PGI2 (mg/mL)</td>
<td>125.57</td>
<td>84.99-275.93</td>
<td>462.9</td>
</tr>
<tr>
<td>NO (U/L)</td>
<td>22.9</td>
<td>16.4-29.0</td>
<td>23.2</td>
</tr>
<tr>
<td>ox-LDL (U/L)</td>
<td>517.2</td>
<td>303.8-1000.0</td>
<td>800.0</td>
</tr>
<tr>
<td>Peroxides (measured by Oxystat) ($\mu$mol/L)</td>
<td>290.5</td>
<td>172.0-428.0</td>
<td>274.0</td>
</tr>
</tbody>
</table>

PGI2 indicates prostacyclin; NO, nitric oxide; and ox-LDL, oxidized LDL
hypertension, coarctation of the aorta, obstructive sleep apnoea, hormonal disorders (like pheochromocytoma, hyperthyreosis, primary aldosteronism, Cushing’s syndrome), or drug-induced hypertension. We also excluded patients with a history of coronary artery disease, heart failure, diagnosed valvular heart disease or cardiomyopathy, as well as patients with diabetes and smokers.

**Endothelial vasodilators and vasoconstrictors in hypertensive patients:** In the hypertensive patients, the level of plasma 6-keto Prostaglandin F\(_1\alpha\), a median prostacyclin metabolite, was significantly low compared to healthy controls (125.57 pg/mL, 25%; 75% quartile range: 84.99; 275.93 and 462.9 pg/mL, 25%; 75% quartile range: 107.69; 849.3, respectively, \(P = 0.009\)). No significant differences according to serum NO (\(P = 0.55\)), oxy-LDL (\(P = 0.24\)) and total peroxide

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>AUC</th>
</tr>
</thead>
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<tr>
<td>Prostacyclin</td>
<td>71.0</td>
<td>66.7</td>
<td>74.6</td>
<td>62.5</td>
<td>0.647</td>
</tr>
<tr>
<td>Oxidized-LDL</td>
<td>67.7</td>
<td>66.7</td>
<td>73.7</td>
<td>60.0</td>
<td>0.366</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>33.9</td>
<td>84.4</td>
<td>75.0</td>
<td>48.1</td>
<td>0.334</td>
</tr>
<tr>
<td>Peroxides (measured by Oxystat)</td>
<td>75.8</td>
<td>33.3</td>
<td>61.0</td>
<td>50.0</td>
<td>0.509</td>
</tr>
</tbody>
</table>

PPV indicates positive predictive value; NPV, negative predictive value and AUC, area under the ROC curve.

**Figure 1.** Receiver operating characteristic (ROC) curves for PGI2, NO, ox-LDL and Oxystat in patients with hypertension.
concentration measured by Oxystat ($P = 0.87$) were found (Table I). PGI2 and oxy-LDL negatively correlated ($r = -0.29$, $P = 0.02$), whereas no statistically significant correlation was observed for NO and Oxystat.

**Diagnostic value of measured parameters in patients with hypertension:** The highest diagnostic sensitivities of all evaluated parameters were found for plasma 6-keto prostaglandin F$_{1\alpha}$ and Oxystat, 71.0% and 75.8%, respectively (Table II). The highest diagnostic specificity and positive predictive values (84.4%, 75.0%, respectively) were found for NO measurements. Specificity for the serum ox-LDL measurements was 66.7%, which was almost the same as that for plasma 6-keto prostaglandin F$_{1\alpha}$ (Table II).

In the ROC analysis (Figure 1), the following cut-off values corresponding to the highest diagnostic accuracy (minimal false-negative and false-positive results) were as follows: 205.69 pg/mL for PGI2, 17.4 U/L for NO, 700 U/L for ox-LDL, and 428 μmol/L for OxyStat. According to these values, the largest area under the ROC curve, 0.647 (95% C.I. 0.549 to 0.737, Figure 1), was found for PGI2. According to the cut-off values derived from the ROC analysis, PGI2 was the parameter that appeared significantly less often in the hypertensive patients when compared to the healthy controls (37.5% versus 62.5%, respectively, $P = 0.0001$).

**Analysis of logistic regression to identify the best diagnostic biomarker in hypertensive patients:** Logistic regression analysis showed that the PGI2 and ox-LDL cut-off values were associated with a 4.9-fold higher significant risk of hypertension (O.R. 4.9 and 4.98, respectively; $P = 0.0008$ and $P = 0.00065$, respectively) (Figure 2).
DISCUSSION

More than 50 million Americans experience systolic hypertension. Hyper-tension is a risk factor for many other vascular diseases including atherosclerosis and stroke. The etiology of essential arterial hypertension remains unexplained.

Endothelial dysfunction occurs in hypertension. The endothelium is now widely considered to be the largest endocrine gland in the human body. Endothelial cells form a barrier between the blood and smooth muscle cells, and play a crucial role in the regulation of vasodilation, hemostasis, angiogenesis, inflammatory processes, and immunology. Mechanical damage, or loss of functional integrity, disturbs the homeostasis of microenvironment, leading to the development of pathological states, like hypertension or atherogenesis. Various vasoactive substances are synthesized in the endothelium. Of these, nitric oxide (NO), prostacyclin (PGI2), and endothelin-1 (ET-1) are the most important. Nitric oxide is a volatile gas continuously produced by endothelial cells that acts to maintain vascular tone.

Nitric oxide synthesized by endothelial nitric oxide synthase (eNOS) plays an important role in the regulation of endothelial function and in the control of blood pressure. However, NO most likely acts in another way. Nitric oxide causes vascular relaxation by increasing intracellular cGMP and activating cGMP-dependent protein kinase I (PKGI).

In our hypertensive patients, the highest diagnostic specificity and positive predictive value were found for NO. However, we still did not find any statistically significant difference according to NO concentrations in patients with arterial hypertension as compared to healthy controls. However, in contrast to NO, significantly lower PGI2 metabolite levels were found in the hypertensive patients. PGI2, another endothelium-derived relaxing agent, is produced by the vascular wall (predominantly by the endothelium) and it acts as a physiological antagonist of TXA2. It is known to cause vasorelaxation and inhibit platelet aggregation by receptor-mediated mechanisms. Moreover, prostacyclin per se is a powerful cytoprotective agent that exerts its action through activation of adenylate cyclase, followed by an intracellular accumulation of cyclic-AMP in various types of cells. In that respect, PGI2 collaborates with the system consisting of NO synthase (eNOS)/nitric oxide free radical (NO)/guanylate cyclase/cyclic-GMP. Both cyclic nucleotides (c-AMP and c-GMP) act in synergy as two energetic fists which defend the cellular machinery from being destroyed by endogenous or exogenous aggressors. While cyclic-AMP is known to act as a second messenger for platelet aggregation, vasorelaxation by hyperpolarization has been described and may provide an explanation for the PGI2 mechanism of action on blood vessels. On the other hand, some data show that prostacyclin, in contrast to NO, does not contribute to the maintenance of basal vascular tone, but its effect on platelets.
is most important. In addition, PGI2 stimulates renin secretion by a direct effect on the juxtaglomerular apparatus, and also has an indirect effect by activating the sympathetic nervous system. Thus, it is useless as an antihypertensive agent. Vascular PGI2 is synthesized endogenously by both endothelial cells and arterial muscular coat. While endothelial cells undoubtedly synthesize larger amounts of PGI2, the musculature comprises a much larger tissue mass so that the overall synthesis is about equally distributed between the endothelial and muscle cells. These differences in synthesis and action between NO and PGI2 may reflect the results in our study. We have proved that PGI2 is an important marker of HTN. In our hypertensive patients, in contrast to NO, PGI2 was significantly decreased, when compared to healthy controls. Among measured parameters, it was the best diagnostic marker of HTN. PGI2 plays a role in the pathophysiology and treatment of pulmonary hypertension. Moreover, in women with pregnancy-induced hypertension and in some patients with essential hypertension, endogenous synthesis of PGI2 has been evaluated by measuring 2,3-dinor-6-keto-PGF1 alpha and has proved to be defective. In the pathophysiology of hypertension during preeclampsia syndrome, the decreased formation of vasodilators such as nitric oxide and prostacyclin may also be involved. However, the role of PGI2 in patients with systemic essential arterial hypertension remains unclear. We have found high diagnostic sensitivity for plasma PGI2 measurements. Considering that our patients were properly treated, it could be summarized that PGI2 is an important and independent marker in hypertensive patients.

Recently, it has become increasingly apparent that endothelial damage may also be effected by enhanced levels of oxidatively modified low-density lipoprotein. The action of oxidative LDL (ox-LDL) on vascular endothelial cells has been proposed to be a crucial process leading to endothelial dysfunction and atherogenesis. However, the biochemical mechanism for such action is not clear. In the present study we therefore aimed to assess the diagnostic role of oxidized LDL, a major pro-atherogenic molecule, in patients with hypertension. Oxidation of low-density lipoprotein (LDL) within the arterial wall is a critical step in atherogenesis. Circulating oxidized LDL does not originate from extensive metal ion-induced oxidation in the blood, but from mild oxidation in the arterial wall by cell-associated lipoxygenase and/or myeloperoxidase. Oxidized LDL induces atherosclerosis by stimulating monocyte infiltration and smooth muscle cell migration and proliferation. Moreover, oxidized LDL inhibits HDL-associated enzyme paraoxonase and PAF-acetyl hydrolase, which circulate in association with HDL and are produced in the arterial wall by macrophages and degraded bioactive oxidized phospholipids. Oxidized LDL acts through the lectin-like receptor for ox-LDL (LOX-1) primarily in the endothelial cells, which allows
the uptake of ox-LDL into endothelial cells. This receptor is highly expressed in blood vessels of animals and humans with hypertension, diabetes mellitus, and atherosclerosis.9 Ox-LDL leads to endothelial activation, dysfunction, and injury. It could be a determinant of endothelium function in coronary arteries.10 Increased LDL oxidation levels were found in young men with borderline hypertension and decreased arterial elasticity. This data may suggest that oxidative modification of LDL particles may play a pathophysiological role in the development of reduced arterial distensibility in hypertension.11 Oxidized-LDL may act synergistically with ET-1 in inducing vascular smooth muscle cell (VSMC) proliferation by way of the activation of redox-sensitive and mitogen-activated protein kinase (MAPK) pathways.12 We assessed the role of ox-LDL and their major oxidative components, measured by Oxystat. We did not find a significant increase in ox-LDL and Oxystat measurements between hypertensive patients and healthy controls. However, we have found a significant negative correlation between ox-LDL and PGI2 levels in patients with HTN. The specificity for the serum ox-LDL measurements was not the highest, but similar to that for plasma PGI2 metabolite. Moreover, the prostacyclin and oxidized-LDL cut-off values were associated with a higher significant risk of hypertension.

Recent studies have showed that various tissues and cells are sensitive to oxidative stress, caused by the formation of free radicals. Thus the determination of oxidative stress is essential in modern medical research and diagnostics. Reactive oxygen species (ROS) influence many physiological processes including host defense, hormone biosynthesis, fertilization, and cellular signaling. In vitro and animal studies suggest that ROS production (termed “oxidative stress”) is associated with endothelial dysfunction. Increased oxidative stress has been implicated in various pathologies, including hypertension, atherosclerosis, diabetes, and chronic kidney disease. A major source for vascular and renal ROS is a family of nonphagocytic NAD(P)H oxidases. Other possible sources include mitochondrial electron transport enzymes, xanthine oxidase, cyclooxygenase, lipoxygenase, and uncoupled nitric oxide synthase. NAD(P)H oxidase-derived ROS plays a physiological role in the regulation of endothelial function and vascular tone and a pathophysiological role in endothelial dysfunction, inflammation, hypertrophy, apoptosis, migration, fibrosis, and angiogenesis, which are important processes underlying cardiovascular and renal remodeling in hypertension and diabetes. These findings have evoked considerable interest because of the possibilities that therapies against nonphagocytic NAD(P)H oxidase to decrease ROS generation and/or strategies to increase nitric oxide (NO) availability and antioxidants may be useful in decreasing vascular injury and renal dysfunction and thereby prevent or regress target organ damage associated with hypertension and diabetes.13 Oxidative stress has a role in the reduction of NO
bioavailability in humans with coronary endothelial dysfunction.\textsuperscript{14} Oxystat is an assay that measures the total peroxide concentration. The results show a direct correlation between free radicals and circulating biological peroxides and thus provide information about oxidative status.\textsuperscript{15,16} Essential hypertension is associated with greater than normal lipoperoxidation and an imbalance in anti-oxidant status, suggesting that oxidative stress is important in the pathogenesis of essential hypertension or in arterial damage related to essential hypertension.\textsuperscript{17} Reduced bioavailability of NO or scavenging of NO through oxidative stress appear to be the key processes through which endothelial dysfunction is manifested in hypertension. The result of an imbalance of counteracting mechanisms, normally designed to maintain vascular homeostasis, leads to vasoconstriction and impaired vascular function. We did not find any diagnostic significance for Oxystat in our study patients.

On the other hand, arterioles within the microcirculation control organ blood flow and represent the main peripheral resistance within the circulation.\textsuperscript{18} Although arterioles have features in common with these conducting vessels, they exhibit distinct properties and the contribution of different pathways to constriction or relaxation varies with vessel size. This is especially the case for endothelium-dependent relaxations. In response to some stimuli, nitric oxide, prostaglandins, and an endothelium-derived hyperpolarizing factor (EDHF) are released from the endothelium. Whereas nitric oxide is dominant in larger vessels, the importance of EDHF increases with decreasing vessel size, like arterioles. However, prostacyclin is known to be more specific for large arteries.\textsuperscript{19} Still, its role in systemic arterial hypertension remains unclear.

Assessing markers of endothelial dysfunction and oxidative stress, we wanted to choose the best diagnostic value in patients with diagnosed arterial hypertension. We wanted to determine the balance between them and if they could influence each other. Zhang, \textit{et al} demonstrated a novel mechanism, reduced l-arginine transport, by which oxidized LDL impairs the ability of the endothelium to generate nitric oxide.\textsuperscript{20} In elderly patients, hypertension was associated with greater than normal levels of lipid oxidation, decreased nitric oxide concentration, and an imbalance in antioxidant status.\textsuperscript{21} Of all measured parameters, we found the best diagnostic significance for PGI2, which also correlated with ox-LDL. Moreover, using these biomarkers to assess endothelial function and oxidative stress could provide insights into the pathways for this vascular disease. Endothelium is thought to be a victim and offender in arterial hypertension; the balance of endothelium-derived factors is disturbed in hypertension. Our results indicate that restoring bioavailability, especially of PGI2, as well as anti-oxidant treatment is able to re-establish this balance and could affect the development of atherosclerosis and cardiovascular events in hypertensive patients.
REFERENCES