Mechanisms of increased vascular superoxide production in human varicose veins

Bartłomiej Guzik1,4, Maciej Chwała3, Paweł Matusik1, Dominik Ludew1, Dominik Skiba1, Grzegorz Wilk1, Wojciech Mrowiecki3, Bogdan Batko1, Andrzej Cencora, Bogusław Kapelak1, Jerzy Sadowski4, Ryszard Korbut2, Tomasz J. Guzik1,2

1 Department of Internal and Agricultural Medicine, Jagiellonian University, Medical College, Kraków, Poland
2 Department of Pharmacology, Jagiellonian University, Medical College, Kraków, Poland
3 Department of Vascular Surgery and Angiology, St. John Grande Hospital, Kraków, Poland
4 Department of Cardiovascular Surgery and Transplantation, Institute of Cardiology, Jagiellonian University, Medical College, John Paul II Hospital, Kraków, Poland

Correspondence to:
Prof. Tomasz J. Guzik, MD, PhD,
Katedra i Klinika Chorób Wewnętrznych i Medycyny Wsi,
Uniwersytet Jagielloński, Collegium Medica, Skarbowc 1,
31-121 Kraków, Poland,
phone: +48-12-633-00-03,
fax: +48-12-631-04-40,
e-mail: tguzik@cm-uj.krakow.pl

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INTRODUCTION Varicose vein disease is one of the most common morbidities in the developed countries. Recent studies have shown that oxidative stress is increased in varicose veins (VV) and venous insufficiency. However, the exact mechanisms of oxidative stress in VV remain unknown.

OBJECTIVES The aim of the study was to measure superoxide anion production and analyze its enzymatic sources in VV in comparison with control human saphenous veins (HSV). Superoxide production was also compared between the proximal and distal segments of the veins.

PATIENTS AND METHODS Proximal and distal segments of varicose veins (14 patients, aged 52 ± 3.5 years) and control veins (15 patients, aged 56 ± 4 years) were obtained during VV removal or elective coronary artery bypass graft surgery, respectively. Subjects were matched for age, sex, and the major risk factors for atherosclerosis. Superoxide was measured by lucigenin-enhanced chemiluminescence (5 µmol/l) in the presence and absence of oxidase inhibitors.

RESULTS Superoxide production was increased in VV compared with control HSV. This increase was particularly evident in the distal segments of VV. There was a significant correlation between superoxide production in the proximal and distal segments of HSV but not of VV. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and uncoupled nitric oxide synthase (NOS) were the major sources of superoxide in VV, because their inhibitors greatly attenuated superoxide production in VV.

CONCLUSIONS NADPH oxidases and NOS could represent valuable drug targets for pharmacological treatment and prevention of varicose vein disease. Oxidative stress may provide a link between endothelial dysfunction, inflammation, and immune activation and the development of chronic venous dysfunction.
unknown. Endothelial dysfunction is one of the possible mechanisms. Recently, it has been suggested that oxidative stress is increased in VV. Increased reactive oxygen species (ROS) production has been implicated in the genesis of endothelial dysfunction characterized by the loss of protective nitric oxide (NO) bioavailability in numerous vascular disease states associated with atherosclerosis, diabetes, hypertension, and smoking. Finally, several recent studies have linked endothelial dysfunction with the development of subsequent venous valve dysfunction, which underlies VV formation. Moreover, oxidative stress, through its effects on matrix metalloproteinases (MMP), could be an important contributor to venous remodeling. ROS cause oxidation of lipid membranes and proteins; they are also critical regulators of inflammation. Moreover, we have previously shown that vascular oxidative stress is a systemic phenomenon related primarily to clinical risk factors. While initial studies have focused on the role of superoxide production and oxidative stress in the arteries, we have shown that it is also very prominent in human veins and may play a role in human pathophysiology.

Despite this evidence, the role of ROS in human varicose vein disease has not been well defined. Two recent studies have shown that oxidative stress is increased in VV, particularly in insufficient vessels. However, they focused primarily on antioxidant enzymes and used indirect and unspecific methods of ROS assessment, such as determination of malondialdehyde (MDA) concentration. While MDA is a product of oxidation by ROS, its generation in biological systems is complex and does not allow for specific identification of individual ROS species involved. Therefore, it is critical to understand which ROS are primarily involved in oxidative stress, and to further define its enzymatic mechanisms in VV. Thus, in the present study we aimed to measure superoxide anion production from varicose vein segments in comparison with nonvaricose human saphenous vein (HSV) samples, obtained from subjects undergoing elective coronary artery bypass grafting (CABG). We also analyzed the enzymatic sources of superoxide production in HSV. Finally, we compared superoxide production in the proximal and distal segments of the veins (control and varicose) in order to gain insight into the role of different degrees of blood reflux and stagnation. This could also shed light on the role of venous pressure increase in the regulation of vascular oxidative stress in VV.

**PATIENTS AND METHODS** Patients and blood vessels

The proximal and distal segments of HSV were obtained from subjects with varicose vein disease (VV; n = 14) undergoing VV removal surgery and from subjects without the disease undergoing elective CABG (excess vein obtained from the proximal and distal vein segments were compared; HSV; n = 15). The proximal segments were typically obtained from the mid thigh, and the distal segments from the calf. Subjects were matched for age, sex, and the major risk factors for atherosclerosis, known from previous studies to affect vascular oxidative stress and superoxide production. Low-molecular-weight heparins, if used, were discontinued 24 hours prior to the surgery. Vessel segments were harvested using a no-touch technique, before surgical distension (HSV) or rapid removal of VV. The segments were immediately transferred to ice cold Krebs-HEPES buffer (99 mmol/l NaCl; 4.7 mmol/l KCl; 1.2 mmol/l MgSO4·7H2O; 1 mmol/l KH2PO4·1.9 mmol/l CaCl2; 25 mmol/l NaHCO3; 11.1 mmol/l glucose; and 20 mmol/l HEPES), delicately flushed, and carefully dissected to remove excess adventitial tissue, using microsurgical instruments. All vessels were collected before topical administration of drugs such as papaverine. The collection of tissue specimens was approved by the Local Research Ethics Committee and informed consent was obtained.

**Vascular superoxide production** Superoxide production was measured by lucigenin-enhanced chemiluminescence (LGCL), using the previously described and validated methods. Briefly, intact vessel segments were equilibrated in Krebs-HEPES gassed with 95%O2/5%CO2 for 30 minutes at 37°C. LGCL from intact vessels was measured in buffer (2 ml) containing low-concentration lucigenin (5 µmol/l). Superoxide production was expressed as relative light units (RLU) per second per mg of dry weight of the vessel (RLU/s/mg dw).

**Determination of the sources of vascular superoxide production** Superoxide release was measured in the presence of various potential oxidase inhibitors to determine its sources in VV. These inhibitors included diphenyliodonium (DPI, 10 µmol/l), flavin oxidase inhibitor such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, apocynin (Apoc, 300 µmol/l; NADPH oxidase inhibitor); oxyxpurinol (Oxy; 100 µmol/l; xanthine oxidase inhibitor); nitro-L-arginine methyl ester (L-NAME; 100 µmol/l; nitric oxide synthase inhibitor); and rotenone (Rot; 100 µmol/l; mitochondrial oxidase inhibitor). As described above, superoxide production was expressed as RLU/s/mg dw.

**Statistical analysis** The results are expressed as means ± standard error of the mean with “n” equal to the number of patients. Statistical comparisons between the 2 groups were made using the t test for independent or dependent variables. P values < 0.05 were considered statistically significant.

**RESULTS** Clinical characteristics of the patients Vessels were obtained from 29 patients (14 subjects undergoing VV surgical removal and 15 control nonvaricose subjects undergoing elective CABG). Demographic and clinical characteristics,
Superoxide production from varicose and nonvaricose veins

Superoxide production, determined by LGCL from intact vascular rings, was observed in all studied vessels and was significantly higher in VV than in the control vessels (Figure 1). This increase was observed in both proximal and distal segments of the vessels. A detailed analysis showed that there was almost a 2-fold increase in basal superoxide production in the distal segments of VV (but not of the nonvaricose control vessels), when compared with the proximal segments (Figure 1).

Specificity for superoxide detection was confirmed by coincubation with superoxide dismutase (SOD). Preincubation with polyethylene glycol-conjugated SOD (500 U/ml), resulted in a very significant inhibition of LGCL signal (92 ± 6% inhibition in control HSV and 93 ± 8% inhibition in VV; n = 5; data not shown).

Relationships between proximal and distal superoxide production

To gain further insight into the differences in superoxide production between the proximal and distal segments of the studied veins, we analyzed the relationship between the absolute amounts of superoxide produced by proximal and distal segments either in the control veins or in VV. Interestingly, superoxide production was very significantly correlated between the proximal and distal vascular segments in nonvaricose veins, but not in VV (Figure 2).

Sources of vascular superoxide production in human varicose disease

To investigate enzymatic sources of superoxide production in VV, we measured superoxide production from distal vascular segments following preincubation with a range of potential oxidase inhibitors (Figure 3). Superoxide production shown in the table, demonstrate that patients were matched for age, sex, and the major risk factors for atherosclerosis known to affect vascular oxidative stress. Patient characteristics and the risk factor profile were typical for patients with atherosclerosis.

### TABLE Major demographic and clinical characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>HSV n = 15</th>
<th>VV n = 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>age, y (mean ± SEM)</td>
<td>56 ± 4.0</td>
<td>52 ± 3.5</td>
</tr>
<tr>
<td>sex (female/male)</td>
<td>9/6</td>
<td>9/5</td>
</tr>
<tr>
<td>risk factors, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>current smoking</td>
<td>6 (40)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>hypertension</td>
<td>9 (60)</td>
<td>8 (57)</td>
</tr>
<tr>
<td>diabetes</td>
<td>2 (13)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>hypercholesterolemia</td>
<td>7 (47)</td>
<td>6 (42)</td>
</tr>
<tr>
<td>medication, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blockers</td>
<td>8 (53)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>acetylsalicylic acid</td>
<td>12 (80)</td>
<td>13 (92)</td>
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<tr>
<td>lipid-lowering agents</td>
<td>10 (66)</td>
<td>10 (71)</td>
</tr>
<tr>
<td>calcium antagonists</td>
<td>6 (40)</td>
<td>5 (35)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>9 (60)</td>
<td>8 (57)</td>
</tr>
</tbody>
</table>

Abbreviations: ACE – angiotensin-converting enzyme, HSV – human saphenous veins, SEM – standard error of the mean, VV – varicose veins

**FIGURE 1** Vascular superoxide anion production in human varicose veins. Superoxide production was measured in paired proximal and distal segments of control, nonvaricose human saphenous vein from patients undergoing CABG (n = 15) and from varicose veins obtained from patients undergoing varicose vein removal (n = 14). Measurements were performed using 5 µmol/l LGCL.

a P < 0.05 vs. HSV

Abbreviations: CABG – coronary artery bypass grafting, dw – dry weight, LGCL – lucigenin-enhanced chemiluminescence, RLU – relative light unit, others – see Table

**FIGURE 2** Relationships between superoxide anion production in the proximal and distal segments of the veins obtained from control subjects with atherosclerosis (A; n = 15) and varicose vein patients (B; n = 14)

Abbreviations: NS – nonsignificant, others – see Table and Figure 1

**FIGURE 3**
FIGURE 3 Enzymatic sources of superoxide anion in human varices. Superoxide production was determined by LGCL (5 μmol/l) in the absence and presence of various oxidase inhibitors. Distal varicose vein segments were incubated for 30 minutes before and during superoxide determination with: diphenyleneiodonium (DPI; 10 μmol/l); apocynin (Apoc; 300 μmol/l); oxypurinol (Oxy; 100 μmol/l); nitro-L-arginine methyl ester (L-NAME; 100 μmol/l); and rotenone (Rot; 100 μmol/l).

Abbreviations: NADPH – nicotinamide adenine dinucleotide phosphate, NOS – nitric oxide synthase, others – see FIGURE 1

production was very significantly inhibited by diphenylene iodonium, an inhibitor of flavin containing oxidases such as NADPH oxidases. Similar degree of inhibition was observed in the presence of apocynin, which is considered a relatively specific inhibitor of NADPH oxidase. Oxypurinol and rotenone had minimal effects on superoxide production from VV. However, the response to inhibition of NOS with L-NAME demonstrated very consistent (about 25%), statistically significant inhibition of superoxide production in all the vessels, indicating important contribution of NOS to oxidative stress rather than NO production in VV.

DISCUSSION We have compared superoxide production between varicose and nonvaricose veins and used the proximal and distal segments of these vessels as a model system to assess the effects of venous pressure increase in the regulation of vascular oxidative stress in human veins. Our study has demonstrated for the first time that VV generate significantly more superoxide anions. Importantly, increased venous blood pressure related to blood reflux and stagnation observed in the distal segments of VV is associated with a further increase in superoxide anion production. At the same time, in nonvaricose veins, with the fully functional venous valve system, superoxide production is not increased in the distal segments of HSV. To gain further insight into the nature (local or systemic) of the factors that regulate superoxide production in VV, we studied the relationships between superoxide production in the proximal and distal vessel segments. Interestingly, while in nonvaricose HSVs there was a strong correlation of superoxide production between the proximal and distal segments, such relationship was not observed in VV. This may indicate that while the systemic factors are most critical in the regulation of superoxide production in nonvaricose veins, in VV the local factors may play an important role that exceeds the effect of systemic regulation of oxidative stress in humans. We have previously identified a systemic nature of both endothelial dysfunction and oxidative stress in patients with atherosclerosis. Endothelial function, superoxide production, and major vascular oxidase expression are all correlated not only between different areas of one vascular bed, but also in functionally distinct vascular compartments, such as the venous and arterial systems. A multivariate analysis performed in these studies showed that the major risk factors for atherosclerosis are the main regulators of systemic vascular oxidative stress. In particular, diabetes and hypercholesterolemia have been defined as the main factors increasing superoxide production in the veins. However, the results of the present study show that apart from systemic effects, local disease environment may be important in enhancing superoxide production and oxidative stress in VV. Lack of correlation between superoxide production in the proximal and distal segments of VV may indicate that such factors as different degrees of blood reflux and stagnation or variable venous pressure have stronger effects than systemic factors. Alternatively to pressure changes, it is possible that certain locally released humoral factors, such as chemokines or cytokines, or certain hypoxia-related metabolites may exert stimulatory effects on ROS production in the distal parts of VV. This may be related to the propagation of VV rather than only to their initiation. Obviously, this hypothesis requires further direct longitudinal studies in larger cohorts, to determine whether local pressure related factors or autacoids play the major role in this disease.

Our study is in line with the previous studies that measured oxidative stress markers in VV and showed a significant increase of MDA, which was also related to venous insufficiency. Krzyściak and Kózka demonstrated increased oxidative stress in insufficient veins and showed that the degree of oxidative damage may correlate with clinical degree of venous disease.

However, our study adds to that knowledge by assessing superoxide production directly from vascular segments, rather than indirectly by determination of lipid oxidation products. Interestingly, previous studies also demonstrated that SOD activity is increased in VV or venous insufficiency. The role of the balance between pro-oxidant and antioxidant enzymes in numerous pathologies has been identified, including cancer, acute coronary syndromes, renal dysfunction, and asthma. While we have not described
that in the present study, we were unable to define clear differences in either SOD activity or expression in human VV (data not shown). This is in line with our previous studies showing that Cu-Zn SOD or MnSOD expression and activity were unchanged in human veins in coronary artery disease.\textsuperscript{25} We did not, however, assess extracellular SOD levels, which could actually be the most important dismutase variant in varicose vein disease.

ROS, and in particular superoxide anions, may play numerous roles in the pathogenesis of chronic venous insufficiency and varicose vein disease.\textsuperscript{10,26}

In general, the current view of the initiation of varicose vein disease include increased destruction of collagen and matrix proteins which are initiated by endothelial dysfunction, characterized by loss of NO and prostacyclin (PGI\(_2\)) bioavailability and subsequently increased vascular inflammation, initiated by increased adherence of leukocytes to venous endothelium.\textsuperscript{5,6,15,27} ROS may regulate these processes on a number of levels (\textbf{FIGURE 4}). First, superoxide anion is the most important cause for biodegradation of NO in human blood vessels.\textsuperscript{28} The reaction of NO with superoxide was experimentally demonstrated for the first time by Gryglewski et al.\textsuperscript{27} This rapid reaction, exceeding Km of any antioxidant enzymes, results in the formation of another strong oxidant, peroxynitrite. We previously described this reaction and its consequences in normal human veins.\textsuperscript{11}

ROS may also directly damage cell lipid membranes.\textsuperscript{30} This leads to concomitant cellular injury of either endothelium or smooth muscle cells, as well as lipid raft dysfunction that may result in vascular and venous valve dysfunction.\textsuperscript{30} ROS are also involved in the induction of inflammatory reactions, thus contributing to the role of immune reactions\textsuperscript{31} that may occur and enhance vascular damage in varicose vein disease. Finally, ROS are potent inducers of MMP. They both increase the activity and expression of MMP-9 and are able to inhibit tissue inhibitors of MMP.\textsuperscript{9,32}

All of these factors may be critical for the initiation of venous dysfunction and may lead to decreased velocity of blood flow through the vein, which further exacerbates the pathological changes. This may lead to venous dysfunction with decreased blood flow. Blood retracts and accumulates in the peripheral segments of the veins leading to venous pressure increase and vascular wall dilation.\textsuperscript{5} Vein tension and hypoxia activate vascular wall cells and infiltration of leukocytes into vascular wall\textsuperscript{29} closing the vicious circle of VV formation. Many of the risk factors known to affect the development of chronic venous insufficiency,
vitamin approach.

Our study, considered together with the previous findings point to the role of ROS in the propagation of varicose disease because their production seems to be further enhanced by local hemodynamic factors.

We have been the first to show the direct measure of superoxide production from VV, but our study has some limitations. First, it would be informative to compare further clinical characteristics of venous functions with measures of superoxide production. Such comparisons, however, would be particularly valuable in larger study populations, where a multivariate analysis would have to be applied to unequivocally identify clinical determinants of superoxide production in VV, which is very important from the clinical point of view. We used LCGL to measure superoxide production. This approach has been criticized in the past, particularly, in relation to the use of high concentrations of lucigenin (above 20 µmol/l) which could artificially enhance superoxide detection through redox cycling. Thus, in the present study, we used low concentrations of lucigenin, which are highly validated measures of superoxide production and are free from the problem of redox cycling.

In summary, the present study has demonstrated for the first time that VV produce increased amounts of superoxide anion compared with normal nonvaricose veins, particularly in the distal segments of the varices. NADPH oxidases are the primary sources of superoxide anion in VV, in line with the earlier findings in normal, control veins from atherosclerotic subjects. Our study, considered together with the previous descriptions of increased oxidative stress in human varicose vein disease, indicates that antioxidant approaches could be valuable in treating venous dysfunction. These could include unspecific approaches, such as N-acetylcysteine or sulodexide treatments, or a particular antioxidant vitamin approach. However, these methods should rather be local and possibly directed towards the inhibition of NADPH oxidase activity in these veins. Further clinical studies are needed to confirm this interesting possibility.

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We dedicate this paper to the memory of one of the creators of the Polish school of phlebology, Professor Andrzej Cencora (1939–2008), who played a critical role in establishing collaborations that led to this study.

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Mechanizmy zwiększonej produkcji anionorodnika ponadtlenkowego w żylakach

Bartłomiej Guzik¹,⁴, Maciej Chwała³, Paweł Matusik¹, Dominik Ludew¹, Dominik Skiba³, Grzegorz Wilk¹, Wojciech Mrowiecki³, Bogdan Batko¹, Andrzej Cencora,
Bogusław Kapelak⁴, Jerzy Sadowski³, Bogdan Batko¹, Andrzej Cencora,
Wojewódzki Oddział Chirurgii Naczyń i Angiologii, Szpital Zakonu Bonifratrów Św. Jana Grandego, Kraków

SŁOWA KLUCZOWE
anionorodnik
ponadtlenkowy, stres oksydacyjny, śródbłonk, wolne rodniki tlenowe, żylaki

STRESZCZENIE

Choroba żylakowa jest jednym z najczęstszych schorzeń w krajach rozwiniętych. Najnowsze badania wykazały, że stres oksydacyjny jest zwiększony w żylach żylakowych (varicose veins – VV) i w przypadku niewydolności żylnej. Jednak szczegółowe mechanizmy stresu oksydacyjnego w VV pozostają nieznane.

Celem pracy był pomiar produkcji anionorodnika ponadtlenkowego i analiza jego enzymatycznych źródeł w żylakach w porównaniu z kontrolnymi ludzkimi żyłami odpiszczelowymi (human saphenous veins – HSV). Porównano również produkcję anionorodnika ponadtlenkowego w segmentach dystalnych żył z jego produkcją w segmentach proksymalnych.

Dystalne i proksymalne segmenty żył żylakowych (14 chorych w wieku 52,3 ±3,5 roku) i kontrolnych (15 chorych w wieku 56 ±4,0 lata) uzyskiwano odpowiednio podczas chirurgicznego usuwania żylaków lub planowych zabiegów pomostowania aortalno-wieńcowego. Pacjenci byli dobrani pod względem wieku, płci i głównych czynników ryzyka miażdżycy. Anionorodnik ponadtlenkowy mieści się w żylach w segmentach proksymalnych.

Produkcja anionorodnika ponadtlenkowego była zwiększona w HSV w porównaniu z kontrolnym HSV. Wzrost ten był szczególnie widoczny w segmentach dystalnych naczyń żylakowych. Znamienna korelacja występowała między produkcją anionorodnika ponadtlenkowego w segmentach dystalnych i proksymalnych HSV, lecz nie HSV. Oksydazy dwunukleotydowego nikotyno-amidenucleotydowego (nicotinamide adenine dinucleotide phosphate – NADPH) i dysfunkcyjna syntaza tlenku azotu (nitric oxide synthase – NOS) były głównymi źródłami anionorodnika ponadtlenkowego w HSV, gdyż ich inhibitory istotnie hamowały produkcję anionorodnika ponadtlenkowego w VV.

Oksydazy NADPH i NOS mogą stanowić ważne cele działania leków w farmakologicznym leczeniu i prewencji choroby żylakowej. Stres oksydacyjny może się okazać elementem łączącym dysfunkcję śródbłonka, stan zapalny i aktywację układu immunologicznego z rozwojem przewlekłej dysfunkcji żylnej.