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Imbalance between the endothelial cell-derived contracting factors prostacyclin and angiotensin II and nitric oxide/cyclic GMP in human primary varicosis

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1 The role of the endothelium in the vasomotor control of human veins in the lower extremity is little understood. We tested the hypothesis that the production of relaxing and contracting factors is altered in endothelial cells from varicose saphenous veins which may predispose to the decreased vessel tone observed in primary varicosis.

2 We determined the intracellular accumulation of guanosine 3′:5′-cyclic monophosphate cyclic GMP; a measure of nitric oxide production) and the release of endothelin and prostacyclin (measured as its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$) from cultured cells derived from the long saphenous veins of patients with primary varicosis (Varicose saphena group, $n=27$) or from patients undergoing coronary artery bypass surgery (Healthy saphena group, $n=22$). In addition, levels of endothelin, angiotensin II, bradykinin, cyclic GMP and cyclic AMP in plasma from patients with primary varicosis and healthy volunteers ($n=8-11$ in each group) were determined.

3 Although basal cyclic GMP levels were similar, more cyclic GMP accumulated in response to histamine ($1-100$ μmol l^{-1}) in cells from varicose saphenous veins (0.75 ± 0.1 pmol per well) than in cells from veins without varicosis (0.27 ± 0.05 pmol per well). Furthermore, the relaxant potency of nitroprusside (1 nmol $l^{-1}-300$ μmol l^{-1}) *in vitro* was higher for varicose veins (mean $EC_{50}=5.9$ μ mol l^{-1}; $n=8$) than healthy veins (mean $EC_{50}=20.0$ μmol l^{-1}; $n=7$).

4 The production of prostacyclin was significantly less in cells from varicose than healthy saphenous veins (66 ± 8.7 and 121 ± 20.1 nmol g^{-1} protein), but the production of endothelin was similar in both groups. Prostacyclin (3 nmol $l^{-1}-30$ μmol l^{-1}) consistently contracted rings of varicose saphenous vein *in vitro* with a mean EC_{50} value of $10-20$ μmol l^{-1} ($n=7$); the maximum tension generated was $\sim$50% of that of a completely depolarizing solution of K^+ (120 mmol l^{-1}).

5 In plasma from patients with varicose veins, levels of cyclic GMP were higher than in healthy controls (9.2 ± 0.03 and 7.2 ± 0.02 nmol l^{-1}), levels of angiotensin II were lower (81 ± 11.5 and 147 ± 21.7 pmol l^{-1}), and levels of endothelin, cyclic AMP, and bradykinin were not different.

6 It is concluded that endothelial cells from diseased saphenous veins secrete less constrictor mediators than cells from healthy veins and that in diseased veins the nitric oxide/cyclic GMP system is up-regulated which may shift the balance of vasoactive factors towards vasodilatation and contribute to the development of primary varicosis.

Keywords: Relaxing and contracting factors; saphenous vein (human); varicosis (primary); vascular tone; endothelial cell culture; umbilical vein (human)

Introduction

Varicose veins of lower limb occur in high prevalence, affecting $\sim$20% of the adult population (Tolins, 1983), and some 25% of patients with leg ulcers and reflux in the deep veins have a primary disorder as a result of a decrease in the venous tone (Clarke *et al*., 1992). With the veins dilated, the cusp will not occlude the lumen, causing valve incompetence, reflux, and high venous pressure (Watts, 1986). A number of changes occur in the microcirculation of the skin of lower limbs, summarized under the heading of venous hypertensive micro-angiopathy.

Phleboedema, induration of tissue in the distal leg, thrombophlebitis and ulcerations are common complications of untreated varicosity (Cornwall *et al*., 1986). The aetiology of primary varicosity is unknown. Several hypotheses have been proposed, including biochemical defects in the collagen structure of the vein wall, separation of vascular smooth muscle cells by fibrous infiltration (Rose, 1986), and presence of arteriovenous shunts (Haimovici, 1987). Hypoxic-induced activation of endothelial cells in venous stasis, followed by synthesis of pro-inflammatory factors and adhesion of polymorphonuclear neutrophils has also been suggested (Michiels *et al*., 1993).

α-Adrenoceptor responsiveness is reduced in varicose saphenous veins (Thulesius *et al*., 1991; Lowell *et al*., 1992) and hand veins of patients with varicosis (Blöchl-Daum *et al*., 1991), which might represent a primary defect resulting in decreased venous tone and predisposing to development of varicose veins. Besides sympathetic activity, endothelium-derived vasoactive mediators are important in controlling local vascular tone (Furchgott & Zawadzki, 1980; Furchgott & Vanhoutte, 1989), including venous tone (De Mey & Vanhoutte, 1982; Monos *et al*., 1995). Two important vasodilators are prostacyclin and nitric oxide, and two vasoconstrictors are endothelin and angiotensin II (Vane *et al*., 1990). Their local levels are controlled by biosynthesis from inactive precursors, mainly in vascular endothelium. This study was designed to test the hypothesis that the balance of the production of vasodilators and vasoconstrictors is disturbed in endothelial cells of human varicose saphenous veins. We cultured endothelial

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cells from healthy and varicose saphenous veins and determined their basal and stimulated nitric oxide (measured as guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP)), prostacyclin (measured as 6-keto-prostaglandin $F_{1\alpha}$) and endothelin production. Furthermore, plasma levels of cyclic GMP, adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP), endothelin and angiotensin II were also measured.

Methods

Origin of endothelial cells

We studied vascular endothelial mediators from 27 patients (21 women and 6 men, mean age 42 ± 2 years) with primary varicosis of the long saphenous vein as confirmed by phlebography, and from a control group (coronary bypass patients; 16 men and 6 women, mean age 65 ± 2 years) without varicosis. Of the subjects with varicosis, 16 were smokers, 4 took oral contraceptives and 6 thyroid hormones; family history showed varicosis in the parents of 10 subjects; 11 suffered from hypercholesterolaemia. Of the control subjects, 9 were smokers, 7 had had a myocardial infarction, 6 were diabetics; they all were taking long-term medication, i.e. (number of patients) β-adrenoceptor blockers (11), isosorbide mononitrate (10), acetylsalicylic acid (9), angiotensin converting enzyme inhibitors (8), nitroglycerin (7), molsidomine (5), nifedipin (4), and isosorbide dinitrate (1). In addition, we measured plasma levels of vasoactive mediators in 11 healthy volunteers (7 women and 4 men, mean age 44 ± 5 years) who were free of any significant previous angiological (phlebological) or other disease, and who were not taking any medication at the time of examination. Lastly, mediator release from endothelial cells derived from umbilical cords of 18 full-term women (mean age 27 ± 2 years) with normal delivery was also studied.

Organ bath experiments

Effect of prostacyclin in varicose veins To determine the mechanical effect of prostacyclin, segments of $\sim 3-4$ cm length of long saphenous vein were taken from the inguinal area and below the knee, respectively of 3 female patients (mean age 43 ± 22 years) undergoing varicose vein stripping, immediately placed in chilled Krebs-Henseleit buffer and rapidly transported to the laboratory (time between excision and begin of experiment: 15–90 min). Veins were superficially cleaned and cut into rings of ~ 4 mm length and the tissues suspended in 5 ml-organ baths for isometric tension measurement. The baths were filled with oxygenated (95% O_2/5% CO_2) warmed (37°C) Krebs-Henseleit solution containing 2.5 mmol l^{-1} Ca^{2+}. After equilibration (1–1.5 h), the contractile response of the rings was tested with phenylephrine (10 μmol l^{-1}; ~ 8 min), the agonist was removed by repeated washes with Krebs-Henseleit buffer, and maximum contractile activity was determined with a depolarizing solution of K^+ (120 mmol l^{-1}; the Na^+ concentration was reduced accordingly). Rings which did not readily contract to depolarization by excess K^+ were considered to be damaged and discarded. Following removal of K^+, baseline tone (almost complete relaxation) was reached within ~ 30 min and the experiment was started. Prostacyclin was added non-cumulatively (due to its quick degradation in warmed solution) at final concentrations ranging from 3 nmol l^{-1} to 30 μmol l^{-1} (~ 1 ng ml^{-1} – ~ 10 μg ml^{-1}), allowing 3 min or attainment of equilibrium for each concentration. At the end of the experiment, K^+ was added once more to establish the maximum contractile response.

Effects of nitroprusside in varicose and non-varicose veins To determine whether responses to nitrovasodilators are increased in varicose saphenous veins, varicose vein segments from 2 female patients (aged 31 and 38 years) and a segment from a male patient with healthy veins (aged 56 years) undergoing coronary bypass surgery were prepared and tested with K^+ as described above (time from operation to start of experiment: 45–100 min) and pre-contracted with a combination of the thromboxane receptor agonist U 46619 (50–100 nmol l^{-1}) (Kühberger *et al*., 1994) and angiotensin II (50–100 nmol l^{-1}). Nitroprusside was added at final concentrations ranging from 1 nmol l^{-1} to 300 μmol l^{-1}, allowing 3 min for each concentration. At the end of the experiment, papaverine (260 μmol l^{-1}) was added to determine maximum relaxation (Kukovetz *et al*., 1979).

Endothelial cell culture

Segments (~ 4 cm) of the inguinal saphenous vein or umbilical vein and their endothelial cells were harvested as described previously (Zilla *et al*., 1990). Cells were suspended in Medium 199 containing human serum (10%) and endothelial cell growth supplement (75 mg l^{-1}) and divided among three wells precoated with human fibronectin (~ 2 cm^2 each). The cells were fed twice a week and allowed to grow to confluence (10–15 days). At this time, the medium was removed from two of the wells and fresh medium was added for 3 h and 21 h, respectively. After this period the medium was removed and immediately frozen at −70°C until analysis of endothelin (3 h incubation) or 6-ketoprostaglandin $F_{1\alpha}$ (21 h incubation). Because rates of mediator release from confluent cells are known to vary with cell density, the two incubations were repeated on the next day, and conditioned media and cells (for determination of cellular protein) were frozen. At this time, the third well was subpassaged, first to one well of a six well plate (~ 9.5 cm^2 per well) and after reaching confluence (4 days), to all 12 wells of two 6-well plates. The cells were grown to confluence (10–14 days) and used for cyclic GMP measurements.

Determination of intracellular cyclic GMP accumulation

Intracellular cyclic GMP accumulation was determined as previously described (Schmidt *et al*., 1989). Briefly, confluent monolayers were washed and equilibrated in 50 mmol l^{-1} HEPES buffer, pH 7.4, containing 3-isobutyl-1-methylxanthine (1 mmol l^{-1}). After 15 min, histamine or nitroprusside was added to give the final concentrations as indicated and reactions were stopped 2 min later by removal of the incubation buffer and treatment for 1 h with 1 ml of 0.01 N HCl. Intracellular cyclic GMP was measured in the supernatants of the lysed cells by radioimmunoassay.

Determination of endothelin and 6-keto-prostaglandin $F_{1\alpha}$ in conditioned medium

The concentration of endothelin was determined essentially as described previously (Brunner, 1995). Briefly, conditioned medium was applied to C2 cartridges, endothelin was eluted with acetonitrile (70%), eluted endothelin was dried and reconstituted for radioimmunoassay by use of a commercial kit (Peninsula; Belmont, CA). The detection limit was 0.05 ± 0.008 fmol per tube ($n = 3$); the cross-reactivity of other endothelin isomers and big endothelin in this assay was less than 5% and 37%, respectively, according to the supplier. 6-Keto-prostaglandin $F_{1\alpha}$ was determined by use of a commercial radioimmunoassay kit (Amersham; Vienna, Austria). The detection limit was 0.45 ± 0.14 fmol per tube ($n = 3$). The samples were appropriately diluted with assay buffer and assayed directly. The cross-reactivity of four other prostaglandins and of thromboxane B_2 in this assay was less than 1% in each case, according to the supplier.

Determination of cyclic GMP, cyclic AMP, angiotensin II, endothelin and bradykinin in plasma

Blood (10 ml) was drawn from the long saphenous vein of patients undergoing varicose vein stripping (7 women and 4 men, age 45 ± 4 years; 3 smokers and 8 non-smokers) and of human volunteers with apparently healthy saphenous veins (7

women and 4 men, age 44±5 years; 4 smokers and 7 non-smokers), the plasma separated and immediately frozen. Blood was drawn from the same anatomical location to avoid mediator metabolism by peripheral organs (De Nucci *et al*., 1988). For both cyclic nucleotides, plasma (0.3 ml) was added to 3 ml trichloroacetic acid (5% v/v), shaken for 25 min, centrifuged, and the supernatant extracted 4 times with 7 ml of diethyl ether for 5 min, the ether was removed and the aqueous sample subjected to radioimmunoassay. For angiotensin II, plasma (1 ml) was diluted 1 : 10 and applied to C2 cartridges, angiotensin II was eluted from the cartridge with acetonitrile (70%), eluted angiotensin II was dried and reconstituted for radioimmunossay by a commercial kit (Amersham; Vienna, Austria). The recovery rate for the extraction procedure was 89±0.9% ($n=3$) as determined by the addition of 3-[^{125}I]-iodotyrosyl^4-angiotensin II. The detection limit was 0.08±0.02 fmol per tube ($n=3$). There was complete cross-reactivity of angiotensin III and less than 2% cross-reactivity of angiotensin I in this assay, according to the supplier. For endothelin, plasma (1 ml) was diluted 1 : 10 and processed as conditioned medium described above. For determination of bradykinin, 2 ml of blood were drawn from the brachial vein of subjects without varicosis (5 men, 3 women, mean age 40±3 years) or subjects with varicosis (3 men, 5 women, mean age 50±4.4 years) into chilled syringes spiked with 0.2 ml of chilled inhibitor cocktail comprising aprotinin (10,000 kallikrein inhibitor units ml^{-1}), soy bean trypsin inhibitor (0.8 mg ml^{-1}, polybrene (4 mg ml^{-1}), 1,10-phenanthroline (10 mg ml^{-1}) and EDTA (20 mg ml^{-1}). The mixture (0.8 ml) was treated with cold ethanol (3 ml), centrifuged, and the supernatant was dried down in a vacuum concentrator. The sediment was taken up in acetone (66%, 0.6 ml), vortexed, petrol ether (1.4 ml) was added, vortexed again, centrifuged, the upper layer removed, and the lower layer dried down for determination of bradykinin by radioimmunoassay as described previously (Miki *et al*., 1996).

Data analysis

All results are given as arithmetic means±s.e.mean. Concentration-effect curves were fitted individually to a Hill type equation yielding (EC_{50} values by use of the GIPMAX curve-fitting programme as described previously (Brunner *et al*., 1991). All data points were given equal weight. For ease of comparison, the data are given as untransformed agonist concentration/tension plots. *n* refers to the number of individuals from whom endothelial cells were taken for culture or, in organ bath experiments, the number of vein rings used (the number of individuals from which they were derived is given in the legends). Data were analysed by Student's *t* test for unpaired observations. A *P* value $\leqslant 0.05$ was considered as significant and is indicated by an asterisk.

Materials

Angiotensin II, U 46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), prostacyclin (Na-salt), 3-isobutyl-1-methylxanthine, soy bean trypsin inhibitor, polybrene, 1,10-phenanthroline and HEPES were purchased from Sigma (Vienna, Austria). All other chemicals were obtained from Merck (Darmstadt, Germany). Aprotinin (trasylol) was a gift from Bayer (Vienna). Endothelial cell growth supplement was from Collaborative Research (Vienna, Austria) and all other tissue culture materials from sources described previously (Kühberger *et al*., 1994).

Results

Validation experiments

Total cellular protein per culture well did not differ for the cells derived from volunteers with healthy ($n=22$) or varicose veins ($n=27$), nor from endothelial cells derived from umbilical veins ($n=15$), indicating that a similar degree of confluence was reached in all cases. The production of mediators per well was similar at confluence and the day after; therefore, both sets of data were combined and expressed as secretion rate based on total cellular protein (data not shown).

Intracellular cyclic GMP accumulation

Formation of nitric oxide by endothelial cells was determined with cyclic GMP as a biochemical marker. In cells from healthy saphenous veins (Figure 1a) basal cyclic GMP level was 0.13±0.01 pmol per well and histamine (1–100 μmol l^{-1}), an endothelium-dependent agonist, increased accumulation up to 0.27±0.05 pmol per well ($n=16$). To determine the responsiveness of (endothelial) soluble guanylyl cyclase, endothelial cells were stimulated with nitroprusside (1 mmol l^{-1}) resulting in a maximum cyclic GMP accumulation of 0.75±0.10 pmol per well. In cells from varicose veins (Figure 1b), basal cyclic GMP content was similar (0.17±0.02 pmol per well), but histamine and nitroprusside increased it to 0.57±0.17 and 1.82±0.38 pmol per well, respectively ($n=20$) ($P<0.05$ in each case vs cells from healthy veins). However, the basal levels of cyclic GMP and the potency of histamine were similar in healthy vein ($EC_{50}=3.3\pm0.8$ μmol l^{-1}) and varicose vein ($EC_{50}=2.4\pm0.6$ μmol l^{-1}; $P>0.05$).

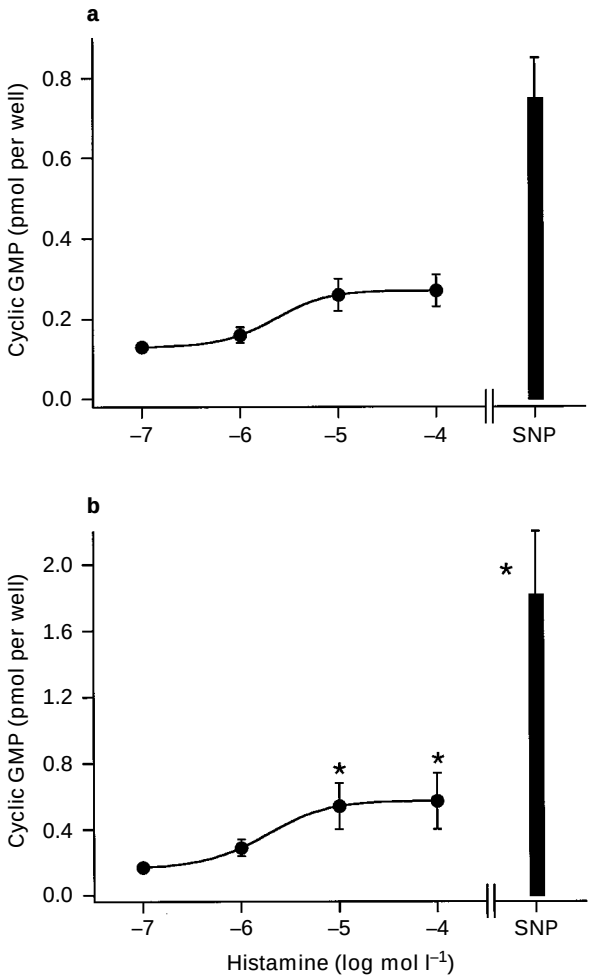

Figure 1 Accumulation of cycic GMP in endothelial cells from healthy (a) or varicose (b) veins in response to histamine or nitroprusside (SNP, 1 mmol l^{-1}). The EC_{50} value for histamine was similar in both cases (~3 μmol l^{-1}), but histamine and nitroprusside produced a greater maximum effect in saphenous vein cells (indicated by the asterisk; $P<0.05$). Mean values±s.e.mean of 16 (a) or 20 (b) cultures are given.

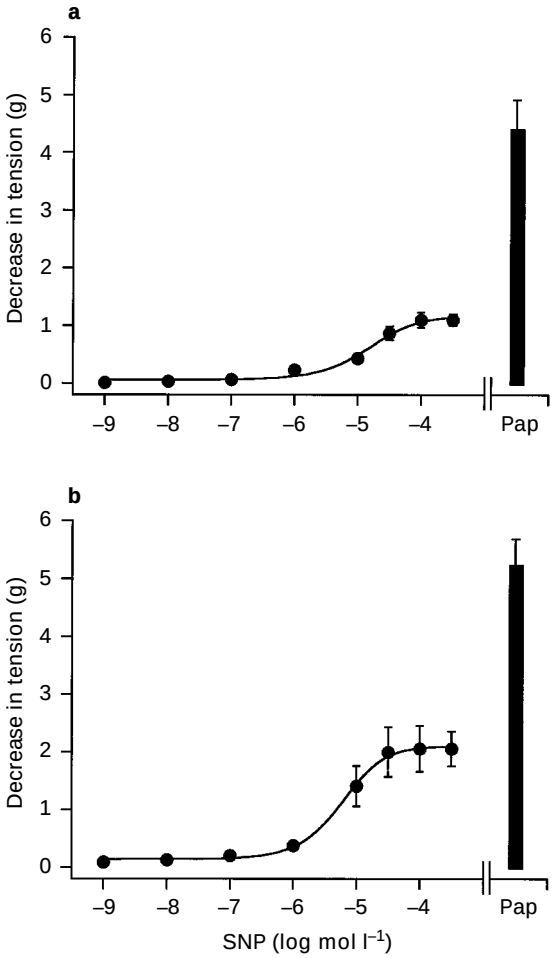

Figure 2 Nitroprusside (SNP)-induced relaxant effects in rings of non-varicose (a), or varicose (b) human saphenous vein. Results are expressed as decrease in tension (g) starting from the tension reached at the end of pre-contraction with U 46619 and angiotensin II (6.8 ± 0.5 g). The agonist was significantly more potent in varicose (mean $EC_{50} = 5.9$ μmol l^{-1}) than non-varicose veins (mean $EC_{50} = 20.0$ μmol l^{-1}). For comparison, the relaxant effect of papaverine (Pap; 260 μmol l^{-1}) is also given. Data points represent the mean $\pm$ s.e.mean of 7 rings derived from 1 patient (a) or 8 rings derived from 2 patients (b) (all from distal vein segments).

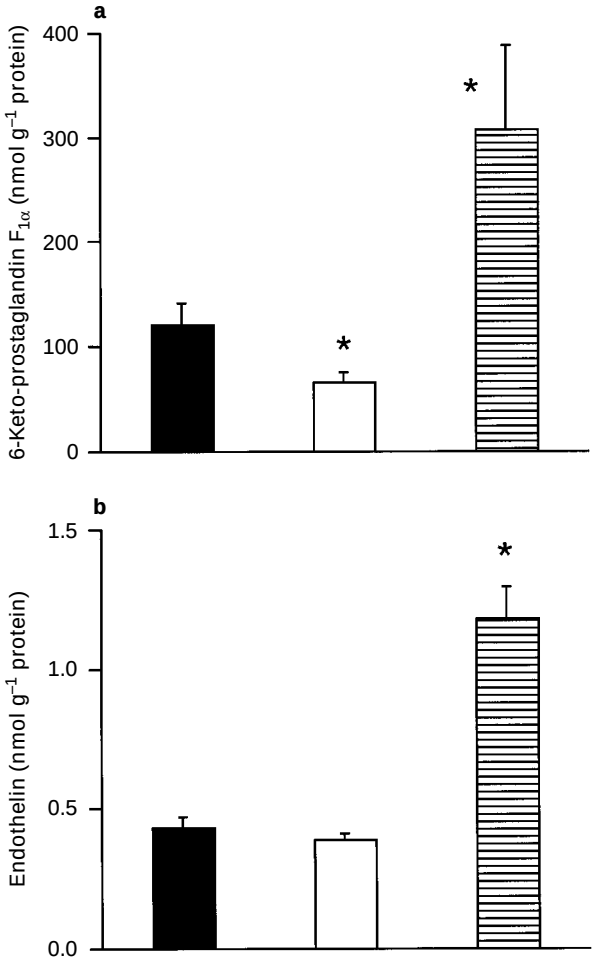

Figure 3 Secretion of prostacyclin (measured as 6-keto-prostaglandin $F_{1\alpha}$) (a) or endothelin (b) by endothelial cells derived from healthy (solid columns; $n = 22$) or varicose (open columns, $n = 27$) saphenous veins, or umbilical cords (hatched columns, $n = 15$). Secretion was monitored over 3 h (a) or 21 h (b) and is given as mean values $\pm$ s.e.mean. *Significantly different from healthy saphenous veins.

Relaxant activity of nitroprusside in healthy and diseased saphenous veins

The response to nitroprusside of saphenous vein rings derived from non-varicose long saphenous veins and patients with varicose veins is shown in Figure 2. The degree of pre-constriction with U 46619 and angiotensin II was the same for healthy and varicose tissues (6.8 ± 0.5 g; $n = 15$). Nitroprusside relaxed all tissues in a concentration-dependent manner but with different potency, i.e. healthy saphenous vein with an EC_{50} value of 20.0 ± 2.5 μmol l^{-1} ($n = 7$; Figure 2a), and varicose vein with an EC_{50} value of 5.9 ± 1.9 μmol l^{-1} ($n = 8$; Figure 2b; $P < 0.05$) (in both cases distal vein segments were used). As to intrinsic activity, the highest concentration of nitroprusside used (0.3 mmol l^{-1}) decreased tension by 1.1 ± 0.13 g in healthy veins and by 2.1 ± 0.40 g in varicose veins ($P = 0.05$). In contrast, papaverine, the most potent smooth muscle relaxant, was similarly effective in healthy and diseased veins (Figure 2).

Mediator secretion into conditioned medium

The release of 6-keto-prostaglandin $F_{1\alpha}$ is shown in Figure 3a. Cells from healthy saphenous veins secreted 121 ± 20.1, and cells from varicose veins secreted 66 ± 8.7 nmol g^{-1} protein ($P < 0.05$). In comparison, cells from umbilical veins secreted 2.6 times as much 6-keto-prostaglandin $F_{1\alpha}$ (308 ± 79.8 nmol g^{-1} protein) as cells from healthy saphenous veins ($P < 0.05$). The release of endothelin is shown in Figure 3b. Cells from healthy and diseased veins produced similar amounts of the peptide (0.43 ± 0.036 and 0.39 ± 0.02 nmol g^{-1} protein; $P > 0.05$), whereas production in umbilical vein endothelial cells was again 2.6 times higher (1.18 ± 0.11 nmol g^{-1}, $P < 0.05$).

Contractile activity of varicose saphenous veins

Figure 4 shows the responsiveness of saphenous vein derived from patients with varicosis towards prostacyclin, phenylephrine and a depolarizing concentration of K^+. Starting from a baseline tension of 0.35 ± 0.01 g, prostacyclin (3 nmol l^{-1}–30 μmol l^{-1}) contracted rings of the vein, taken either proximally or distally, with similar potency (EC_{50} values: 20.5 ± 2.7 and 14.1 ± 4.1 μmol l^{-1}, $n = 6$ and 8, respectively; $P > 0.05$). However, the maximum force generated by 30 μmol l^{-1} prostacyclin was higher in proximal (5.5 ± 0.93 g) than distal tissues (3.5 ± 0.51 g; $P < 0.05$). Similarly, a submaximum concentration of phenylephrine (10 μmol l^{-1}) generated a higher tension in proximal vein (14.3 ± 2.0 g) than distal vein (5.8 ± 1.1 g; $P < 0.05$), and a similar ratio was obtained for K^+ (Figure 4).

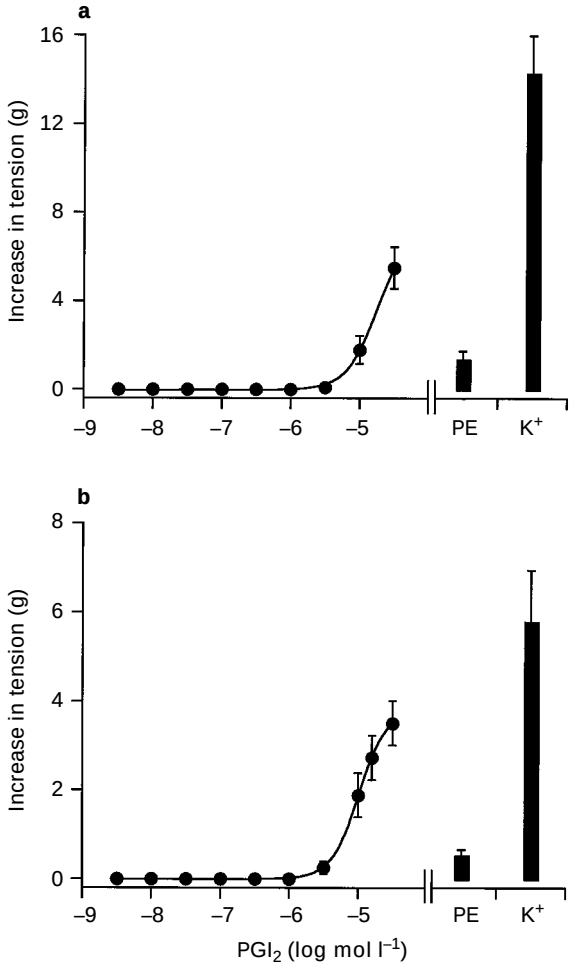

Figure 4 Prostacyclin (PGI_2)-induced contractile effects in rings of human proximal varicose (a) and distal varicose (b) saphenous vein. All rings were stably relaxed (baseline tension: 0.35 ± 0.01 g) when prostacyclin was added. Results are expressed as increase in tension (g) above baseline. For comparison, the contractile effects of phenylephrine (PE, 10 μmol l^{-1}) and K^+ (120 mmol l^{-1}) are also given. Data points represent the mean $\pm$ s.e.mean of 6 rings derived from 2 patients (a), or 8 rings derived from 2 patients (b).

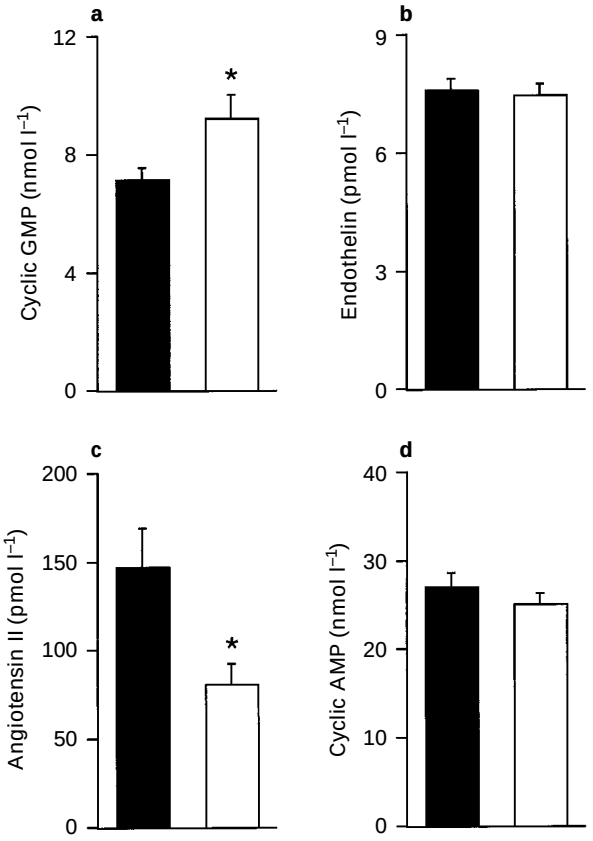

Figure 5 Plasma levels of cyclic GMP (a), endothelin (b), angiotensin II (c) and cyclic AMP (d) measured in subjects with healthy (solid columns, $n = 11$) or varicose (open columns, $n = 11$) saphenous veins. Mean values $\pm$ s.e.mean are given. *Significant difference between groups.

Mediator levels in plasma

Figure 5 shows the plasma levels of cyclic nucleotides, endothelin, and angiotensin II in volunteers with non-varicose veins and patients with varicose saphenous veins ($n = 11$ in each case). The cyclic GMP concentrations were lower in subjects with healthy saphenous veins (7.1 ± 0.4 nmol l^{-1}) than in varicose patients (9.2 ± 0.8 nmol l^{-1}; $P < 0.05$); the endothelin and cyclic AMP levels were similar in both groups (7.6 ± 0.3 and 7.5 ± 0.3 pmol l^{-1}, 27.1 ± 1.5 and 25.1 ± 1.2 nmol l^{-1}; $P > 0.05$ in each case), and the angiotensin II concentration was higher in subjects with healthy veins (147 ± 21.7 pmol l^{-1}) than in those with varicose veins (80 ± 11.5 pmol l^{-1}; $P < 0.05$), but the bradykinin levels (obtained from the brachial vein) were not different (13.3 ± 0.9 vs 12.8 ± 2.3 pmol l^{-1}, $n = 8$; data not shown).

Discussion

The present study is the first demonstration of impaired release and altered plasma levels of endothelial relaxing and contracting factors in patients with primary varicosis. We have found that conditioned medium exposed to primary culture endothelial cells derived from varicose veins accumulated significantly less 6-keto-protaglandin $F_{1\alpha}$ than cells obtained from non-varicose veins, and that the local levels of cyclic GMP were higher, and of angiotensin II were lower in patients with varicose veins, whereas secretion and plasma level of endothelin were not affected. Collectively, these changes may predispose to a reduced venous tone in patients with varicosis.

The relaxant effect of nitric oxide on smooth muscle are mediated by increases in cyclic GMP (Moncada *et al*., 1991), and in vascular preparations extracellular levels of cyclic GMP correlate well with intracellular levels (Rapoport & Murad, 1983). Like their arterial counterparts, venous endothelial cells are fully functional with respect to nitric oxide synthesis and release (Ignarro *et al*., 1987), and veins, including human saphenous veins, show nitric oxide-dependent vasodilatation (Yang *et al*., 1991; Chua *et al*., 1993; Higman *et al*., 1993). Little is known about the role of nitric oxide in human primary varicosis. In the present study we have found that endothelial cells from varicose saphenous veins synthesized greater amounts of cyclic GMP when stimulated with histamine or a high concentration of nitroprusside than cells from non-varicose subjects (Figure 1). Similarly, guanylyl cyclase activity of saphenous vein smooth muscle was higher in varicose than healthy tissues, as judged by the four times lower EC_{50} value for relaxant activity of nitroprusside in organ bath experiments (Figure 2). The latter also showed a similar capacity for relaxation, irrespective of anatomical origin of the vein (proximal vs distal, not shown) and the cellular mechanism involved (cyclic GMP in the case of nitroprusside, and cyclic AMP for papaverine). Together, these data support the view that, in human varicose veins, endothelial production of nitric oxide is activated and the sensitivity of venous smooth muscle is increased which, individually or in combination, may then lead to increased synthesis of cyclic GMP in smooth muscle cells and heightened vascular relaxation. However, the actual pro-

relaxant pathophysiological stimuli are unknown. We also found that levels of cyclic GMP in plasma, sampled directly from the saphenous vein, were higher in patients with varicosity than subjects with healthy veins, which agrees with the higher biosynthetic capacity for cyclic GMP of endothelial cells. In spite of this, the exact relationship between altered plasma levels and endothelial production of cyclic GMP in subjects with normal and varicose saphenous veins cannot be ascertained from this study, but would require sampling of blood from the proximal saphenous vein as well as the corresponding arterial location. Like other vascular tissues, saphenous veins produce prostanoids, of which prostacyclin appears to be the most abundant (Monos *et al*., 1995). In the present study, we observed a smaller rate of release of 6-keto-prostaglandin $F_{1\alpha}$ from cells derived from diseased veins compared to healthy veins. The significance of this finding was ascertained by functional experiments *in vitro* with isometric tension measurements. Prostacyclin consistently contracted varicose saphenous veins in concentration-dependent fashion, clearly supporting the present premise that prostacyclin exerts constrictor activity in this vessel. The prostanoid was similarly potent in proximal and distal saphenous vein segments, but the proximal tissues developed a higher tension upon depolarization with K^+ (Figure 4). Our findings corroborate and extend previous results of one group in which prostacyclin was found to contract normal saphenous veins, although few quantitative data were given (Sinzinger & Fitscha, 1984; Sinzinger & Feigl, 1985). Moreover, canine pulmonary and femoral veins also appear to be contracted by prostacyclin (Miller & Vanhoutte, 1985). The prostacyclin production of segments of varicose veins has been found to be reduced (Biagi *et al*., 1988), increased (Haynes *et al*., 1990), or similar (Sinzinger & Feigl, 1985) to that of healthy veins. These inconsistencies may be due to different methods of tissue sampling and handling which may massively alter *in vitro* prostanoid production and are avoided in the present study. We have found no difference in cyclic AMP levels in saphenous vein plasma from individuals with healthy and varicose veins, indicating that increased cyclic AMP production may not be involved in varicosity.

The peptide endothelin (Yanagisawa *et al*., 1988) is synthesized in arterial and venous endothelial cells and is a potent vasoconstrictor and pressor agent in animals and man (Masaki, 1995). Endothelin elicits its effects via specific membrane receptors, ET_A and ET_B, and is more potent and effective as a constrictor of isolated veins than of isolated arteries. In view of the sensitivity of human saphenous veins to exogenous endothelin and the presence of ET_A and ET_B receptors, both mediating vasoconstriction in these vessels (White *et al*., 1994), the assessment of the release of endothelin from saphenous veins was of particular interest. Our findings that neither the rates of endothelin synthesis and release by cultured cells nor the plasma levels differed in the two study groups make it unlikely that this mediator plays a role in the development of varicosity. Rather, angiotensin II may be of importance. Angiotensin converting enzyme activity (which results in angiotensin II formation) was demonstrated in endothelial cells as well as the adventitia of different large vessels, including human saphenous vein (Rogerson *et al*., 1992). This activity persisted after removal of the endothelium and was mostly associated with the vasa vasorum in these vessels (Rogerson *et al*., 1992). Therefore, we determined this mediator in the plasma of saphenous veins, which may better represent its total local production. The predominant venoactive effect of antiotensin II is constriction, either directly via angiotensin II receptors (Smith & Timmermans, 1994), and/or possibly indirectly via activation of the sympathetic and endothelin systems. In this light, the reduced angiotensin II plasma level we found in the group with varicosity may predispose to a lowered tone of the saphenous vein wall and contribute to the development of the disease. Also, the lower production of angiotensin II by varicose veins may be at the origin of the reduced, or lost, contraction-facilitating effect of the endothelium of varicose vein segments stimulated with noradrenaline (Thulesius *et al*., 1991). On the other hand, bradykinin levels were not increased in the varicose vein group as would be expected when angiotensin converting enzyme activity is reduced, which may be due to a compensatory degradation by other mechanisms. Further studies of endothelial angiotensin converting enzyme activity, angiotensin II formation, release and action are warranted to substantiate a causal role of angiotensin II in primary varicosity.

Finally, we would advise caution in the use of umbilical vein endothelial cells as controls. We found a much higher mediator release in these cells than in cells from healthy saphenous veins, as also shown previously in a comparison between human late pregnancy decidual and umbilical vein endothelial cells (Gallery *et al*., 1995). This casts doubt on the validity of their widespread use as a general surrogate for endothlial cells.

In conclusion, the present study shows that cultured endothelial cells derived from human varicose saphenous veins secrete less 6-keto-prostaglandin $F_{1\alpha}$ and angiotensin II, two vasoconstrictor factors in this vessel, than cells derived from healthy saphenous veins. The imbalance may result in reduced active wall tension due to weak smooth muscle contraction, and may constitute an important factor in chronic venous insufficiency. These data significantly extend previous evidence implicating endothelial dysfunction in primary varicosity (Biagi *et al*., 1988; Blöchl-Daum *et al*., 1991; Thulesius *et al*., 1991; Lowell *et al*., 1992) and may have important therapeutic consequences for the treatment of this disease.

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