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Effects of hydroxyethylrutosides on hypoxia-induced activation of human endothelial cells *in vitro*

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1 A clinically available mixture of hydroxyethylrutosides (HR) was examined as inhibitors of endothelial cell activation by hypoxia *in vitro*. Thus, the effects of HR on ATP depletion, phospholipase A_2 activation and neutrophil adherence were investigated in hypoxia-activated human umbilical vein endothelial cells in primary cell culture.

2 Our results show that HR inhibited two important steps of the activation of endothelial cells by hypoxia: the decrease in ATP content, which is the starting point of the process, and the activation of phospholipase A_2 one enzyme responsible for the release of inflammatory mediators. This inhibition was dose-dependent with 70 to 90% inhibition at 500 $\mu g\,ml^{-1}$ of HR.

3 In addition, hypoxia-activated endothelial cells increased their adhesiveness for neutrophils. This process could also be prevented in a dose-dependent manner if endothelial cells were incubated in the presence of HR. This inhibition was confirmed by a morphological study.

4 In conclusion, the results of this study suggest that a possible explanation for the improvement in venous insufficiency by HR observed clinically could be their ability to inhibit the activation of endothelial cells during blood stasis.

Keywords: Hydroxethylrutosides; endothelial cells; hypoxia; phospholipase A_2; neutrophil adhesion; ATP

Introduction

Venous insufficiency is a common disease of which varicose veins represent one important aspect. Amongst the therapeutic approaches available, phlebotonic drugs are proposed in order to prevent the worsening of the disease. Hydroxyethylrutosides (HR) is a standardized mixture of semisynthetic flavonoids which has been shown to improve lower limb venous insufficiency in double-blind studies performed with objective and subjective criteria (Balmer & Limoni, 1980; Pulvertaft, 1983; Neumann & van den Broek, 1990). HR are known to reduce the capillary filtration rate in patients with venous insufficiency (Roztocil *et al.*, 1977; Cesarone *et al.*, 1992), to decrease microvascular permeability in several experimental models (Gerdin & Svensjo, 1983; Kendall *et al.*, 1993) and to reduce oedema formation (Rehn *et al.*, 1991). All these effects may explain the beneficial effect of HR in patients with venous insufficiency.

Recently, a new hypothesis has been proposed for the development of varicose veins: during blood stasis, ischaemic conditions develop leading to a strong activation of the endothelium. This activated endothelium releases growth factors for smooth muscle cells (Michiels *et al.*, 1994b) and inflammatory mediators resulting in the recruitment, adherence and infiltration of polymorphonuclear neutrophils (PMN) (Arnould *et al.*, 1993; 1994). Both effects lead to alterations in the venous wall similar to the ones observed in varicose veins (Michiels *et al.*, 1994a).

We wanted to see whether HR could inhibit this deleterious cascade and in this way, explain its positive effect *in vivo* in patients with venous insufficiency. For this purpose, we used an experimental model wherein human endothelial cells are incubated under hypoxia *in vitro*. In these conditions, a strong activation of these cells is first observed which starts with a decrease in the adenosine 5'-triphosphate (ATP) content of the cells. This is followed by an increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$ (Arnould *et al.*, 1992), an activation of phospholipase A_2 (PLA_2), an increase in the prostaglandin (PG) synthesis (Michiels *et al.*, 1993), a stimulation of platelet-activating factor (PAF) synthesis and an increased adhesiveness of endothelial cells for unstimulated polymorphonuclear neutrophils (PMN) (Arnould *et al.*, 1993). HR were tested in these conditions and their effects are presented.

Methods

Human umbilical vein endothelial cells isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe *et al.* (1973b). Cords were stored at 4°C just after birth in stock solution (4 mM KCl, 140 mM NaCl, 10 mM HEPES, 1 mM glucose, 100 $mg\,ml^{-1}$ streptomycin, 100 μml^{-1} penicillin and 0.25 $mg\,ml^{-1}$ fungizone, pH 7.3). Cords were rinsed with 20 ml phosphate-buffered saline (PBS) containing antibiotics and fungizone at concentrations aforementioned. Umbilical veins were incubated 35 min at 37°C with 4 ml collagenase type II 0.05% in PBS. Collected cells were then seeded in M199+20% foetal calf serum (FCS) (Gibco, Praisley, Scotland), centrifuged 10 min at 1000 r.p.m. and cultured in 0.20% gelatin-coated culture dishes (Falcon Plastics, Oxnard, CA). The day after, cells were washed with medium in order to eliminate blood cell contamination. Only monolayers of primary cultures that were tightly confluent were used for these studies. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen assessed by immunofluorescence staining (Jaffe *et al.*, 1973a).

In vitro *model of hypoxia*

Ischaemia was simulated by exposing cells to hypoxia (100% N_2) at 37°C. Cells were seeded in gelatin-coated Petri dishes ($\phi = 35$ mm, Falcon Plastics, Oxnard, CA). For incubation, cells were rinsed twice with HBSS and covered with 0.7 ml of HBSS for hypoxia incubation. Medium was reduced to a uniform thin layer to decrease the diffusion distances of the atmospheric gases. Hypoxia was produced with an atmo-

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sphere of 100% N_2 in an incubator gas chamber while the control cells were kept in normal atmosphere containing 20% O_2 Po_2 in the medium was 130 mmHg in normal conditions and dropped to 10 mmHg after 15 min hypoxia as described previously (Michiels *et al.*, 1992). Hypoxia at 120 min was chosen because it is the maximal hypoxia time that endothelial cells can sustain without loss of viability. In order to avoid the reoxygenation effect, assays were performed immediately after the hypoxia incubation, i.e. in less than 1 min for measurements of arachidonic acid (AA) release and ATP content.

Cell viability

The evaluation of the toxicity of the drug was first made on cells seeded in multidish (5,000 cells/well, 96-well plate, Corning, Corning, NY) and incubated in normal atmosphere (95% air + 5% CO_2) during 5 days. The toxicity was estimated by the determination of the cells still attached to the substrate by a DNA assay using propidium iodine. In brief, cells were rinsed twice with phosphate buffered saline (PBS), permeabilized with 50 μl ethanol for 30 min at room temperature and then labelled with 100 μl propidium iodine at 10 $\mu g\,ml^{-1}$ in PBS. Fluorescence was measured with an excitation wavelength of 530 nm and an emission wavelength of 620 nm in a cytofluorimeter (CytoFluor 2300, Millipore, Paris, France).

ATP assay

Fifty thousand endothelial cells were seeded in Petri dishes ($\phi = 35$ mm). ATP assay was performed using a bioluminescent ATP assay kit (FL-ASC; Sigma, St Louis) with luciferine and luciferase. To ensure reproducibility and low background, all the technical precautions described in the corresponding manual sheet were observed. Human umbilical vein endothelial cells (HUVEC) were exposed to hypoxia, rinsed with PBS and permeabilized with 1 ml of the 'somatic cell releasing reagent' (Sigma) also found in the kit for a few seconds. The lysates were collected at 4°C. ATP assay was performed just as indicated by Sigma on a luminometer (Lumac Biocounter 2010, Switzerland). A standard curve was also performed and results were expressed as fmol ATP.

[^{3}H]-arachidonic acid release

Fifty thousand endothelial cells were seeded in Petri dishes ($\phi = 35$ mm) and radiolabelled with 0.25 $\mu Ci\,ml^{-1}$ [^{3}H]-arachidonic acid ([^{3}H]-AA) for 18 h. After this time, the cells were washed 3 times with 1 ml HBSS, then 0.7 ml HBSS was added and cells were incubated under hypoxia as described earlier. After incubation, the media were collected and counted for 3 min in a liquid scintillation counter after the addition of 5 ml Aqualuma (Lumac, Landgraaf, The Netherlands). The total radioactivity was obtained by lysis of non-incubated cells with 0.7 ml NaOH 0.5 N. The recovery of radioactivity was always higher than 95% when supernatant and lysate from incubated cells were counted separately. The percentage of fatty acid release was calculated as: 100 × number of d.p.m. in the extracellular fluid/total number of d.p.m. in the lysate of non-incubated cells (Godfrey *et al.*, 1987).

Isolation and labelling of human neutrophils

Human PMN were purified from the blood of healthy donors by the procedure of Boyum (1976). In brief, 30 ml of venous anticoagulated blood were mixed with 5 ml of 6% dextran and allowed to sedimentate at 20°C for 60 min. After hypotonic lysis of erythrocytes performed with NaCl 0.2% for 1 min, cells were centrifuged 20 min at 1,000 r.p.m. on Lymphoprep (Nycomed Pharma, Oslo, Norway). For labelling, cells at a density of 5×10^6 cells ml^{-1} were incubated with 20 μCi $^{51}Cr\,ml^{-1}$ in HBSS without calcium and magnesium for 1 h at 37°C and then washed three times before use.

Adhesion assay

Endothelial cells were seeded at confluence (40,000 cells per cm^2) in Petri dishes ($\phi = 35$ mm). After hypoxia incubation, HBSS was removed and 1 ml of ^{51}Cr-labelled PMN (5×10^6 cells ml^{-1}) was added on the endothelial cell monolayer. After a 5 min co-incubation of PMN with endothelial cells (37°C), dishes were washed three times with 0.5 ml of HBSS to remove non-adherent cells. The remaining adherent cells were then solubilized with 0.5 ml NaOH 1N and the radioactivity was measured in a gamma-counter.

Reagents

Modified Hanks' balanced salt solution (composition, mM: NaCl 140, KCl 5, $MgSO_4.7\,H_2O$ 0.4, $MgCl_2 \cdot 6\,H_2O$, 0.5. Na_2HPO_4. $2H_2O$ 3, KH_2PO_4 0.4, glucose 5.5, pH 7.35) containing $CaCl_2$ 1 mM (HBSS) was prepared in our laboratory. HR (O-(β-hydroxyethyl)rutosides licensed under the name of Venoruton/Paroven were generously given by Zyma Benelux (Brussels, Belgium). HR are a standardized mixture of semi-synthetic flavonoids comprised mainly of mono- (about 5%), di- (about 34%), tri- (about 46%) and tetra- (about 5%) hydroxyethylrutosides. HR were directly dissolved in HBSS and then serially diluted in HBSS. The flavonoid the most abundant in the HR mixture is the trihydroxyethylrutoside (46%) which has a molecular weight of 790: 100 $\mu g\,ml^{-1}$ of this molecule thus gives a concentration of 126 μM. The chemicals of analytical grade were from Merck (Darmstadt, Germany).

Statistics

The data are presented as means ± 1 s.d. Analysis of variance (ANOVA 1) was performed and means were compared one to another by Scheffé's contrasts.

Results

Effects of HR on HUVEC growth

The influence of HR on cell growth in normal culture conditions (20% O_2) was first tested in order to determine the *in vitro* toxicity of this compound on human endothelial cells. HUVEC were incubated for 5 days in the presence of different concentrations of HR and cell growth was evaluated by the DNA content of the cultures. In these experimental conditions, the assay also tests for a possible inhibition of cell growth by the drug. We observed that concentrations higher than 2,000 $\mu g\,ml^{-1}$ of HR induced a decrease in cell content. Significant 11% and 14% decreases in cell content were observed respectively at 2,000 and 4,000 $\mu g\,ml^{-1}$ of HR (data not shown). Lower concentrations were then selected for the further experiments.

Effect of HR on hypoxia-induced HUVEC activation

The first parameter that changes during hypoxia incubation is the ATP content of the cells: the ATP content linearly decreases during the hypoxia incubation (Arnould *et al.*, 1992). Figure 1 shows that hypoxia led to a 43% decrease in ATP content after 2 h. When the hypoxia incubation was performed in the presence of different concentrations of HR, a strong protection was observed which was dose-dependent between 50 and 1,000 $\mu g\,ml^{-1}$. Significant 48% and 99% inhibition were observed respectively at 100 and 1,000 $\mu g\,ml^{-1}$ HR. However, at the highest concentration (1,500 $\mu g\,ml^{-1}$ of HR), this inhibition was lower (36%).

Since PLA_2 is responsible for the synthesis of inflammatory mediators such as prostaglandins and platelet-activating factor, we tested whether HR could also prevent its activation during hypoxia. This enzyme cleaves arachidonic acid (AA) present in *sn*-2 position of membrane phospholipids. PLA_2

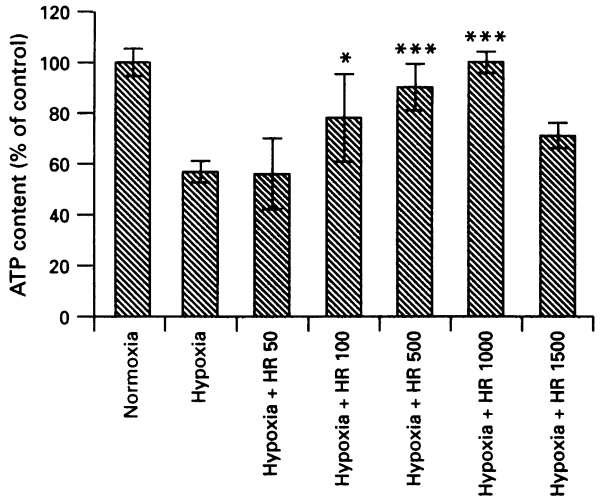

Figure 1 Effect of hydroxyethylrutosides (HR) on the hypoxia-induced decrease in ATP concentration. HUVEC seeded at 5,000 cells per cm^2 were incubated for 2 h under hypoxic conditions in the presence or in the absence of different concentrations of HR expressed in $\mu g\,ml^{-1}$. Control cells were incubated for 2 h in normoxic conditions. ATP concentration was assayed in HUVEC by the luciferine-luciferase system. Results are expressed as means ± s.d. for 2 experiments performed in triplicate ($n=6$). Significantly different from cells incubated under hypoxia in the absence of HR with $^{*}P<0.05$, $^{***}P<0.001$, by use of analysis of variance with Scheffé's contrasts.

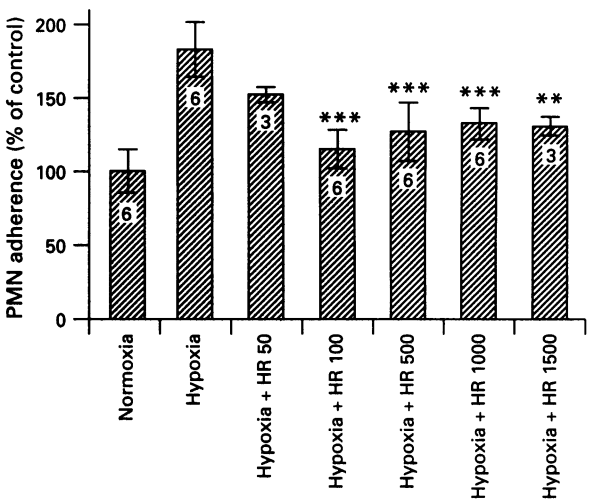

Figure 3 Effect of hydroxyethylrutosides (HR) on the hypoxia-induced PMN adherence on endothelial cells. HUVEC monolayers (40,000 cells per cm^2) were incubated for 2 h under hypoxic conditions in the presence or in the absence of different concentrations of HR expressed in $\mu g\,ml^{-1}$. Control cells were incubated for 2 h in normoxic conditions. One ml containing 5×10^6 ^{51}Cr-labelled PMN was added just after these incubations for 5 min. Results are expressed as means ± 1 s.d. (the number of replicates is indicated in the columns). Significantly different from cells incubated under hypoxia in the absence of HR with $^{**}P<0.01$, $^{***}P<0.001$, using an analysis of variance with Scheffé's contrasts.

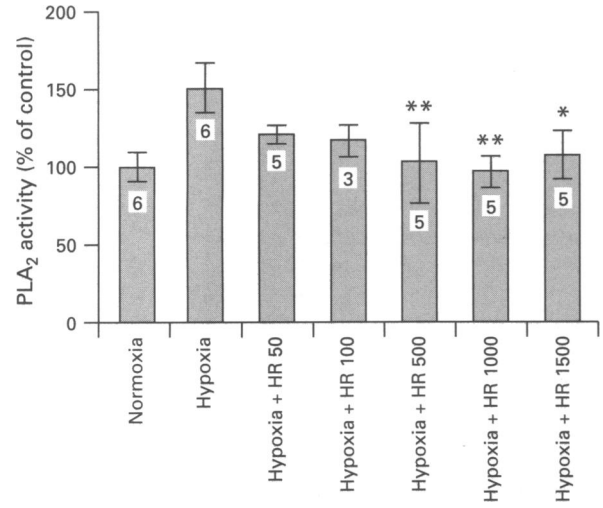

Figure 2 Effect of hydroxyethylrutosides (HR) on the hypoxia-induced activation of phospholipase A_2 (PLA_2). HUVEC were seeded at 5,000 cells per cm^2 and labelled with [^{3}H]-AA for 18 h. After being rinsed, they were incubated for 2 h under hypoxic conditions in the presence or absence of different concentrations of HR expressed in $\mu g\ ml^{-1}$ and PLA_2 activity was estimated by the release of [^{3}H]-AA in the medium during these incubations. Control cells were incubated for 2 h in normoxic conditions. Results are expressed as means ± s.d. (the number of replicates is indicated in the columns). Significantly different from cells incubated under hypoxia in the absence of HR with $^{*}P< 0.05$, $^{**}P<0.01$, $^{***}P<0.001$, by use of analysis of variance with Scheffé's contrasts.

activity can indirectly be estimated by measuring the release of tritiated AA from prelabelled phospholipids. An incubation of 2 h of hypoxia increased the activity of PLA_2 by 50% (Figure 2). This hypoxia-induced increase in activity was inhibited when HUVEC were incubated in the presence of HR in a dose-dependent manner between 50 and 1,000 $\mu g\,ml^{-1}$ of HR. Significant 65% and 100% inhibition were observed respectively at 100 and 1,000 $\mu g\,ml^{-1}$ of HR.

When HUVEC were incubated under hypoxia during 2 h, PMN adherence greatly increased mainly due to the increased synthesis of platelet-activating factor. Two hours of hypoxia led to an increase of 80% of PMN adherence to HUVEC (Figure 3). This adherence was highly inhibited when HUVEC were incubated under hypoxia in the presence of HR. This inhibition was significant between 100 and 1,500 $\mu g\,ml^{-1}$ of HR with a maximum inhibition of 81% at 100 $\mu g\,ml^{-1}$ HR. The hypoxia-induced increase in PMN adherence is well illustrated in the micrographs presented in Figure 4 as well as its marked inhibition in the presence of HR. These morphological observations confirm the biochemical data and are in accordance with previous data (Michiels *et al.*, 1993).

The different effects of HR on the hypoxia-induced decrease in ATP content, activation of PLA_2 and increase in PMN adherence are summed up in Figure 5 which presents the percentage of protection obtained with different concentrations of HR on these three parameters. A fair dose-dependent parallel inhibition between 50 and 1,000 $\mu g\,ml^{-1}$ HR was observed on the decrease in ATP content and the activation of PLA_2. This protection was limited at higher concentrations (1.5 mg ml^{-1}) probably due to the toxic effect of the compound. Hypoxia incubation was performed in a saline solution which probably increased the toxic effect of HR described in complete medium. The inhibitory effect of HR on the hypoxia-induced increase in PMN adherence occurred within the same range of concentrations but peaked at lower concentrations and seemed more sensitive to the toxic effects of HR. Such an interference of the toxicity of HR on PMN adherence to hypoxic EC was even stronger when the experiment was performed in other experimental conditions (data not shown).

Discussion

HR are widely used in the treatment of chronic venous insufficiency and a recent meta-analysis of 15 double-blind clinical trials showed that HR therapy gave significantly superior beneficial effects compared to placebo in the treatment of symptoms related to this disease (Poynard & Valterio, 1994). Besides their effects on capillary filtration, micro-

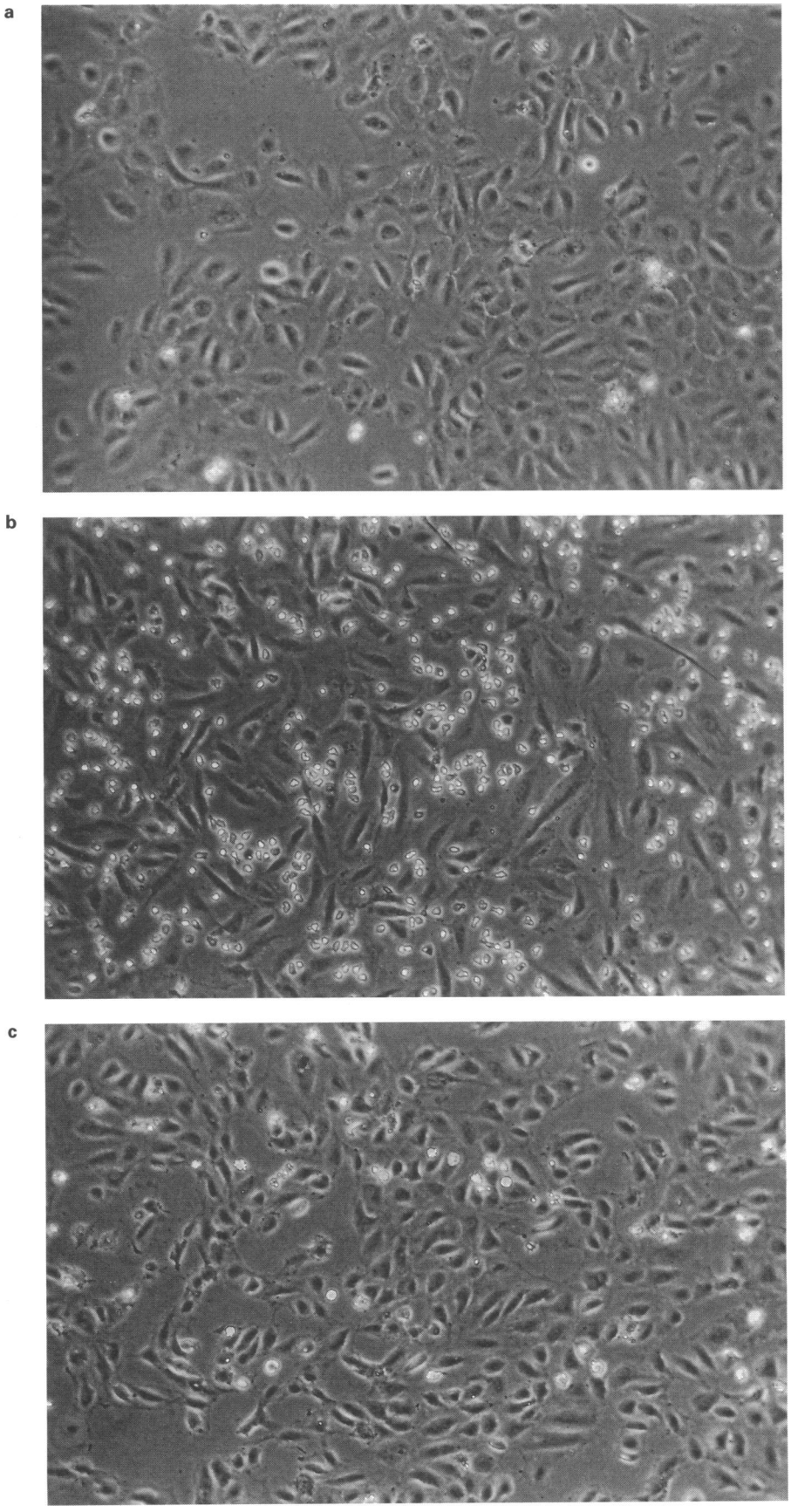

Figure 4 Micrographs in phase contrast microscopy of PMN adherent to endothelial cells. HUVEC monolayers were incubated for 2 h in normoxic conditions (a) or in hypoxic conditions in the presence (c) or in the absence (b) of 1,000 $\mu g\,ml^{-1}$ hydroxyethylrutosides (HR); then unstimulated PMN (5×10^6 cells) were added for 5 min and 4 washes were performed to eliminate non-adherent PMN (magnification = × 144).

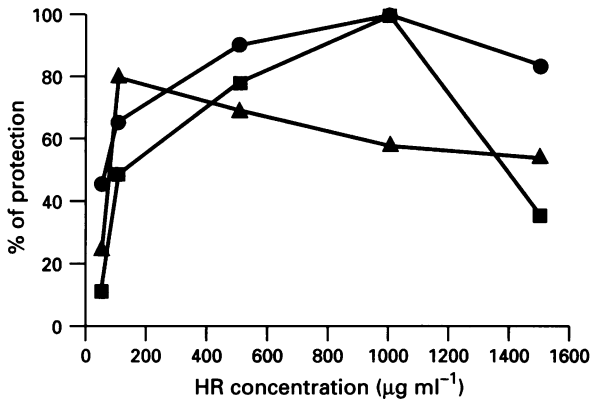

Figure 5 Effect of different concentrations of hydroxyethylrutosides (HR) on the hypoxia-induced decrease in ATP content (■), activation of phospholipase A_2 (PLA_2) (●) and increase in PMN adherence (▲). Results are expressed as % of protection and are means from 2 experiments.

vascular permeability and oedema formation, the exact mechanism of action of this drug is still unclear. The drug has also been shown to increase the $TcPO_2$ in patients with chronic venous insufficiency (Neumann & van den Broek, 1990) which has been suggested to be related to the ability of HR to enhance tissue fibrinolysis and thus reduce the pericapillary fibrin cuff (Quigley & Faris, 1991). However, the role of this fibrin cuff by limiting oxygen diffusion in the aetiology of chronic venous insufficiency is far from being demonstrated and remains very controversial (Scurr & Coleridge-Smith, 1994).

One parameter which could influence the development of chronic venous insufficiency is the trapping of white cells in capillaries, resulting in an ischaemic situation which would lead to tissue alterations (Coleridge-Smith *et al.*, 1988). In addition, recent experimental findings suggest structural damages secondary to chronic tissue hypoxia in varicose veins (Crotty, 1992). Michiels *et al.* (1994a) recently proposed that activation of the endothelium by hypoxic conditions occurring during blood stasis could be a possible initial factor for the development of varicose veins. Hypoxia-activated endothelial cells release growth factors for smooth muscle cells and inflammatory mediators which induce the recruitment and activation of neutrophils; all of these events eventually lead to a pathological vessel wall with features similar to those observed in the varicose vein wall.

In this study, we have shown that HR were able to inhibit in a dose-dependent manner the activation of HUVEC by hypoxia as shown by the inhibition of the decrease in ATP content and of the activation of PLA_2 as well as the resulting adherence of neutrophils. Because the range of inhibition was approximately the same (from 70 to 90% at 500 $\mu g\,ml^{-1}$ HR) for all these processes, it seems that the action of HR is due to the inhibition of the initiating process which leads to the activation of endothelial cells, i.e. the decrease in ATP concentration. That HR can prevent such a cascade occurring during blood stasis is important because it explains the inhibition of the infiltration of recruited PMN in the underlying tissue; this would thus block the subsequent local inflammation and the appearance of oedema. HR would also prevent PMN plugging in the microcirculation because the protected endothelium remains non-adhesive for PMN, thus facilitating their circulation in the vessels. HR have indeed been found to be clinically useful in improving the microcirculatory flow and the oxygen exchange (Neumann & van den Broek, 1990) which may be beneficial in improving symptoms.

HR have already been shown to have a protective effect on endothelium *in vivo* by attenuating the nicotine-induced increase in circulating endothelial cells (Prerovsky & Hladovec, 1979). There is also evidence that the HR molecules localize on the endothelial cells of the venous walls of patients with varicose veins (Neumann *et al.*, 1992; Ekestrom & Welti, 1990).

The strong protective effects, shown in this study with HR, on endothelial activation by hypoxia and on PMN adherence fit well with the clinical observations of the protective effects of the drug. It probably represents one of the molecular and cellular mechanisms underlying the drug protection on the venous wall and in this way gives a rationale for our understanding of the preventive action of phlebotonic drugs.

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