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European Journal of Pharmacology 315 (1996) 227–233

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Effect of aescine on hypoxia-induced activation of human endothelial cells

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Received 12 February 1996; revised 31 July 1996; accepted 6 August 1996

Abstract

Phlebotonic drugs are very often old drugs which improve symptoms in chronic venous insufficiency but their precise mechanism remains unclear. One reason for this lack of information is our poor understanding of the aetiology of the varicose vein. One hypothesis which is being more and more substantiated is that the origin of the disease lies in the activation of the endothelium during blood stasis, leading to a cascade of reactions which, in the long term, alter the structure of the vein wall. In this work, we tested aescine (Reparil i.v. form), a phlebotonic drug, in an in vitro model which mimics this situation, i.e. human endothelial cells exposed to hypoxic conditions. Aescine was shown to inhibit 2 important steps of the activation of endothelial cells incubated 120 min under hypoxia: the decrease in ATP content, which is the starting point of the activation cascade, and the increase in the activity of phospholipase A_2, an enzyme responsible for the release of precursors of inflammatory mediators. Hypoxia-activated endothelial cells also increase their adhesiveness for neutrophils. This process could also be prevented in a dose-dependent manner if endothelial cells were incubated in the presence of aescine. This inhibition was confirmed by morphological observations in scanning electron microscopy. All 3 effects were already evidenced at 100 ng/ml and were maximal at 750 ng/ml. These effects obtained at very low concentrations probably represent one of the main molecular and cellular mechanisms that underlie, among others, protection of the vessel wall. Objective criteria for our understanding of the preventive action of this phlebotonic drug are, thus, provided.

Keywords: Cell activation; Hypoxia; Endothelium; Neutrophil adherence; Aescine; Reparil

1. Introduction

Venous insufficiency is a common disease and the appearance of varicose veins is one of its main pathological expression. Among the therapeutic tools available are the phlebotonic drugs which are proposed to prevent worsening of the disease. Reparil is one of these drugs whose active molecule is aescine, a triterpenic glucoside. It is used in the treatment of chronic venous insufficiency disease (Wolfram, 1968; Hübner, 1985, 1987). This drug was tested in pharmacological studies on animals and was shown to have anti-inflammatory activity (Rothkopf et al., 1980), to prevent edema formation (Makrigiannis, 1969; Berberich, 1968; Hefti and Kappeler, 1975) by reducing microvascular permeability, and to exert a powerful venotonic activity by increasing venous tone (Annoni et al., 1979). Aescine is known to reduce the capillary filtration rate in patients with venous insufficiency, to decrease microvascular permeability in several experimental models and to reduce edema formation (Hefti and Kappeler, 1975). All these effects may explain the beneficial effect of aescine in patients affected by venous insufficiency. However, its precise mechanism of action remains poorly understood.

In veins, endothelial cells constitute the main cellular barrier separating blood and tissues and any defect in their integrity or homeostasis may lead to edema formation, inflammation and loss of venous tone. Recently, a new hypothesis has been postulated to explain the development of varicose veins: during blood stasis, ischemic conditions occur, leading to strong activation of the endothelium. It was observed that the decrease in oxygen supply when endothelial cells are incubated under hypoxia leads to a decrease in the ATP content due to a decrease in mitochondrial oxidative phosphorylation which cannot be compensated for by glycolysis (Janssens et al., 1995). This

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PII S0014-2999(96)00645-0

decrease in ATP content induces a serie of cellular modifications, such as an increase in cytosolic calcium concentration (Arnould et al., 1992), the release of growth factors for smooth muscle cells (Michiels et al., 1994a) and of inflammatory mediators, like prostaglandins (Michiels et al., 1993) and platelet-activating factor (Arnould et al., 1993), resulting in the recruitment, adherence and activation of polymorphonuclear neutrophils (Arnould et al., 1993, 1994). This cascade of events accounts very well for the alterations observed in varicose veins (Michiels et al., 1994b).

In this work, we investigated whether aescine could inhibit this deleterious cascade and whether we could in this way, explain its positive effect in vivo in patients suffering from chronic venous insufficiency. For this purpose, we used the experimental model with human endothelial cells incubated under hypoxia in vitro. Under these conditions, strong activation of these cells is observed during the first 2 h of incubation as described here above. Aescine was tested in this experimental model and its effects on ATP content, phospholipase A_2 activity and neutrophil adherence were evaluated. The study was designed in order to find if protection against hypoxia of endothelial cells by aescine could be obtained.

2. Materials and methods

Aescine is the active molecule of Reparil from Madaus (Köln, Germany). Aescine is a triterpenic glucoside ($C_{55}H_{87}O_{24}$) present as the sodium salt form in the Reparil (i.v. form) used in this study. The drug was solubilised directly in the incubation buffer.

2.1. Human umbilical vein endothelial cells isolation and culture

Human umbilical vein endothelial cells were isolated according to Jaffe et al. (1973a). 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 streptomycin, 100 U/ml penicillin and 0.25 μg/ml fungizone, pH 7.3). The cords were rinsed with 20 ml phosphate buffer saline (PBS) containing antibiotics and fungizone at the above concentrations. Umbilical veins were incubated 35 min at 37°C with 4 ml collagenase type II (Sigma, St. Louis, MO, USA) 0.05% in PBS. The cells collected were then seeded in M199 + 20% fetal calf serum (Gibco, Paisley, UK), centrifuged 10 min at 1000 rpm and cultured in 0.20% gelatin-coated culture dishes (Falcon Plastics, Oxnard, CA, USA). The next day, the cells were washed with medium in order to eliminate blood cell contamination. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen by immunofluorescence staining (Jaffe et al., 1973b).

2.2. In vitro model of hypoxia

Ischemia was simulated by exposing cells to hypoxia (100% N_2) at 37°C. Cells were seeded in gelatin-coated Petri dishes (ø = 35 mm, Falcon Plastics). For incubation, the cells were rinsed twice with modified Hanks balanced salt solution containing 1 mM $CaCl_2$ (HBSS) and were covered with 0.7 ml of HBSS for hypoxia incubation. The medium was reduced to a uniform thin layer to decrease the diffusion distances of the atmospheric gases. Hypoxia was produced with an atmosphere of 100% N_2 in an incubator gas chamber while the control cells were kept in a normal atmosphere containing 20% O_2. pO_2 in the medium was 130 mmHg under normal conditions and dropped to 10 mmHg after 15-min hypoxia and remained at 10 mmHg throughout the incubation as previously described (Michiels et al., 1992). Hypoxia for 120 min was chosen because it is the maximal hypoxia time that endothelial cells can sustain without loss of viability. In all experiments for hypoxia incubation, the assays were performed immediately after the hypoxia incubation in order to avoid reoxygenation.

2.3. Cell viability

The toxicity of the drug was evaluated with cells seeded in a multidish (64 000 cells/well, 24-well plate; Corning, NY, USA) and incubated in normal room air (95% air + 5% CO_2) for 2 h in HBSS. After 24 h, the medium was removed and 1 ml of HBSS containing or not the drug at different concentrations was added to each well. The lyophilyzed drug was directly solubilised in HBSS. Toxicity was estimated from the number of cells still attached to the substrate after 2, 4 and 6 h by in situ protein assay according to Lowry et al. (1951).

2.4. ATP assay

For ATP assay with a bioluminescent ATP assay kit (FL-ASC; Sigma) using luciferine and luciferase, 50 000 endothelial cells were seeded in Petri dishes (ø = 35 mm). To ensure reproducibility and low background, all the technical precautions described in the instructions supplied with the kit were observed. Human umbilical vein endothelial cells were exposed to hypoxia, rinsed with PBS and permeabilized with 1 ml of the 'somatic cell releasing reagent' (Sigma) for a few seconds. The lysates were collected at 4°C. ATP was assayed, as indicated by Sigma, on a luminometer (Lumac Biocounter 2010, Switzerland). The results were expressed as percentages of the number of control cells incubated 2 h under normoxic conditions.

2.5. [^{3}H]Arachidonic acid release

First, 50 000 endothelial cells were seeded in Petri dishes (ø = 35 mm) and radiolabeled with 0.25 μCi/ml

[^{3}H]arachidonic acid for 18 h. The cells were then washed 3 times with 1 ml HBSS. HBSS (0.7 ml) was then added and cells were incubated under hypoxia. After the 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 Nederlands). The total radioactivity was obtained by lysis of non-incubated cells under hypoxia but labeled with [^{3}H]arachidonic acid for 18 h with 0.7 ml NaOH 0.5 M. 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 dpm in the extracellular medium/total number of dpm in the lysate of non-incubated cells (Godfrey et al., 1987). The percentage of arachidonic acid incorporation was very reproducible and ranged between 20 and 25%.

2.6. Isolation and labeling of human neutrophils

Human polymorphonuclear neutrophils were obtained from the blood of healthy donors by the procedure of Boyum (1976). Briefly, 30 ml of venous anticoagulated blood was mixed with 5 ml of 6% dextran and allowed to sediment at room temperature for 60 min. After hypotonic lysis of erythrocytes with NaCl 0.2% for 1 min, the cells were centrifuged 20 min at 1000 rpm on Lymphoprep (Nycomed Pharma, Oslo, Norway). For labeling, neutrophils at density of 5×10^6 cells/ml were incubated with 20 μCi ^{51}Cr/ml in HBSS without calcium and magnesium for 1 h at 37°C and then washed 3 times before use.

2.7. Adhesion assay

Endothelial cells were seeded at confluence (40 000 cells/cm^2) in Petri dishes (ø = 35 mm). After hypoxia incubation, HBSS was removed and 1 ml of ^{51}Cr-labeled neutrophils (5×10^6 cells/ml) was added on the endothelial cell monolayer. After a 5-min co-incubation with endothelial cells (37°C), the dishes were washed 3 times with 0.5 ml of HBSS to remove non-adherent neutrophils. The remaining adherent neutrophils were then solubilised with 0.5 ml NAOH 1 M and the radioactivity was measured in a gamma-counter.

2.8. Statistical analysis

The data are presented as mean ± S.D. For each kind of experiments, 3 independent experiments in triplicate (n = 9) were performed, with different cell cultures from different umbilical veins. Under these conditions, we have to take into account the inherent variability between the different primary cultures. The analysis of variance with 2 crossed factors (ANOVA-2), one which is random (the experiment number) and the other which is fixed (the type of treatment) was used to test for the equality of means. If the ANOVA-2 showed a similarity between means of each experiment but a global difference between means of each treatment, the means for each treatment were compared one to each other by Scheffé's contrasts and all values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Toxicity of aescine for endothelial cells

Due to the lack of data from in vitro studies with aescine and in order to choose a non-toxic concentration range, we first tested the influence of aescine on cell toxicity. Human umbilical vein endothelial cells were incubated 2, 4 and 6 h in HBSS in the presence of different concentrations of aescine. Cell toxicity was evaluated from the protein content of the cultures. While in situ protein measurement is not the best way to measure toxicity, it does give rapid and reliable data on important toxicity effects. Based on these data, a non-toxic drug concentration range between 1 and 750 ng/ml was determined and selected for subsequent experiments. It must be noted that aescine as well as other drugs tested under our experimental conditions seems to be more toxic for cells incubated under stress conditions, such as hypoxia that under control conditions. Indeed, we did not observe toxicity of aescine when endothelial cells were incubated with aescine at 1000 ng/ml for 120 min when measured by protein content (respectively, 18.77 ± 1.15 mg/well vs. 20.28 ± 3.78 mg/well for control cells (n = 3)). However, at this concentration, the ATP content after 120 min hypoxia represented 38.68 ± 11.07% of the control cells incubated under normoxia (100 ± 11.33%) when the hypoxic cells without aescine still contain 66.41 ± 15.06% of ATP (n = 3). These results suggest that at 1000 ng/ml, aescine was toxic for endothelial cells incubated under hypoxia even if the protein content was unaffected.

3.2. Effect of aescine on hypoxia-induced decrease in ATP concentration

Before cell death can be induced by oxygen deprivation, hypoxia is able to strongly activate the endothelial cells. This effect was observed during the first 2 h of hypoxia. The first parameter that changes during hypoxia incubation is the cellular ATP content: the ATP content decreases linearly during the hypoxia incubation (Arnould et al., 1992). Fig. 1 shows that hypoxia led to a 40% decrease in ATP content after 2 h. When the hypoxia incubation was performed in the presence of different concentrations of aescine, strong protection was observed which was dose-dependent. Increasing protection (between 38 and 95%) was observed, respectively, between 100 and 750 ng/ml of aescine. A possible effect of aescine under normal oxygen tension was also tested. The ATP content

of cells incubated under normoxia with aescine (750 ng/ml) represents 97.9 ± 12.3% ($n = 3$) of the control values (100 ± 4.1%, $n = 3$). This result supports the conclusion that the molecule has no effect on the energy metabolism of cells kept under normoxia for 2 h. The 100% control value is given for the ATP content of cells incubated 120 min in normoxic conditions. The ATP content of these control cells was 15.0 ± 1.7 nmol/mg of proteins ($n = 3$). This concentration is in perfect agreement with the concentration reported by Watanabe et al. (1991) for aortic endothelial cells of rats: 16.0 ± 1.9 nmol/mg of proteins.

3.3. Effect of aescine on hypoxia-induced increase in phospholipase A_2 activity

The decrease in the ATP content during hypoxia is followed by an increase in cytosolic calcium concentration which in turn activates phospholipase A_2 (Michiels et al., 1993). Since phospholipase A_2 is responsible for the synthesis of inflammatory mediators, such as prostaglandins and platelet-activating factor, we tested whether aescine could also prevent its activation during hypoxia. This enzyme cleaves arachidonic acid in the *sn*-2 position of membrane phospholipids. Phospholipase A_2 activity can be estimated indirectly by following the release of [^{3}H]arachidonic acid from pre-labeled phospholipids. Incubation for 2 h under hypoxia increased the activity of phospholipase A_2, which was 1.9-fold higher than that in the control (Fig. 2). The hypoxia-induced increase in phospholipase A_2 activity was strongly inhibited when endothelial cells were incubated with 100 ng/ml or more aescine. Significant inhibitions between 57 and 72% were observed for concentrations between 100 and 750 ng/ml of aescine.

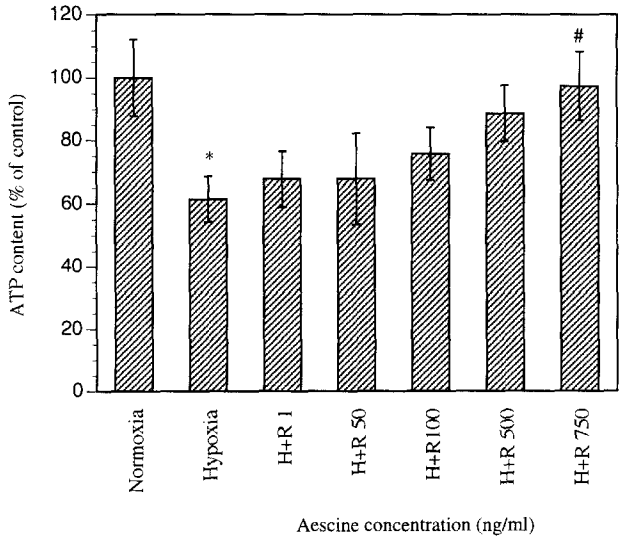

Fig. 1. Effect of aescine on the hypoxia-induced decrease in ATP content. Endothelial cells seeded at 5000 cells/cm^2 were incubated for 2 h under hypoxia in the presence or in the absence of different concentrations of the drug. Control cells were incubated 2 h under normoxic conditions. ATP concentration was assayed in cells by the luciferine-luciferase system. Results are expressed in % of control as mean ± S.D. for 3 experiments in triplicate ($n = 9$). # Significantly different from cells incubated 2 h under hypoxia without aescine, with $P < 0.05$; * significantly different from control normoxia with $P < 0.05$ using an ANOVA-2 with Scheffé's contrasts.

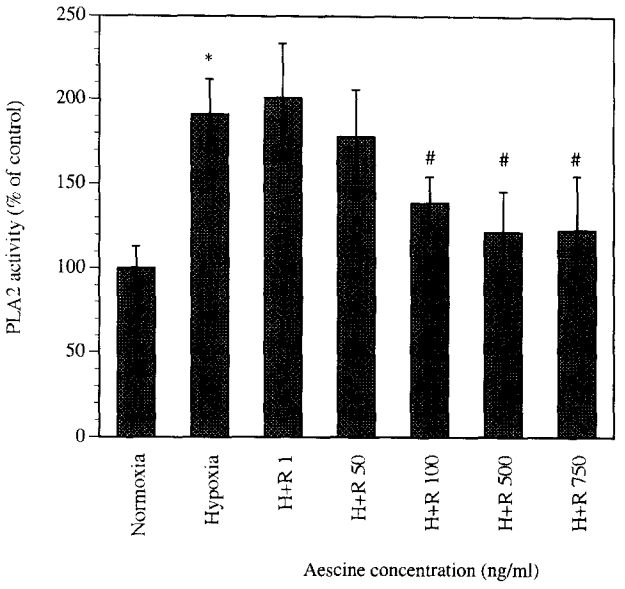

Fig. 2. Effect of aescine on the hypoxia-induced activation of phospholipase A_2. Endothelial cells were seeded at 5000 cells/cm^2 and labeled with [^{3}H]arachidonic acid for 18 h. Cells were incubated 2 h under hypoxia in the presence or in the absence of different concentrations of the drug, and phospholipase A_2 activity was estimated from the release of [^{3}H]arachidonic acid in the medium. Control cells were incubated 2 h under normoxic conditions. Results are expressed in % of control as mean ± S.D. for 3 experiments in triplicate ($n = 9$). # Significantly different from cells incubated 2 h under hypoxia without aescine, with $P < 0.05$; * significantly different from control normoxia with $P < 0.05$ using an ANOVA-2 with Scheffé's contrasts.

3.4. Effect of aescine on hypoxia-induced increase in neutrophil adherence

Adherence of unstimulated neutrophils to endothelial cells is low under normal conditions but increases when either neutrophils or endothelial cells are activated. When endothelial cells were incubated under hypoxia for 2 h, their adhesiveness increased greatly (Fig. 3). This adherence was strongly inhibited when endothelial cells were incubated under hypoxia in the presence of aescine. Protection was significant between 500 and 750 ng/ml of aescine with a very strong inhibition already observed at 100 ng/ml (62%).

In order to support the premise that the observed decrease in ATP content induced by hypoxia is the initial event, resulting in an increase in neutrophil adherence, experiments were performed in order to decrease the ATP content of endothelial cells by means other than hypoxia and to test the subsequent effect on neutrophil adherence.

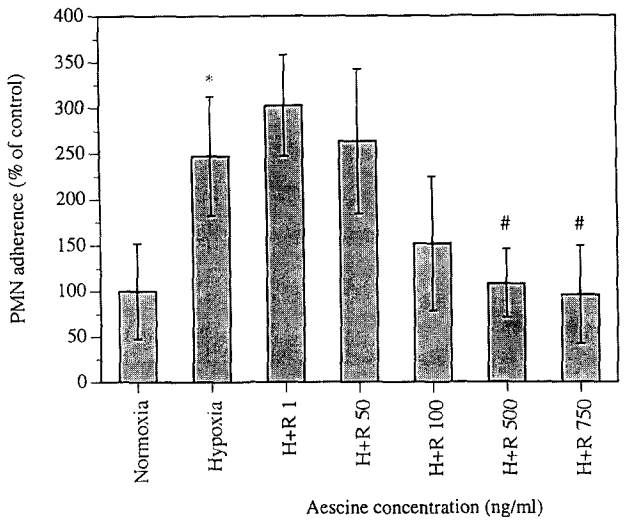

Fig. 3. Effect of aescine on hypoxia-induced neutrophil adherence on endothelial cells. Cells seeded at 40000 cells/cm^2 were incubated 2 h under hypoxia in the presence or in the absence of different concentrations of the drug before adding 1 ml of labeled neutrophil suspension (5×10^6 cells/ml). Control cells were incubated 2 h under normoxic conditions. Results are expressed in % of control as mean ± S.D. for 3 experiments in triplicate ($n = 9$). * Significantly different from cells incubated 2 h under hypoxia without aescine with $P < 0.05$; # significantly different from control normoxia, with $P < 0.05$ using an ANOVA-2 with Scheffé's contrasts.

To decrease ATP content, we chose 'chemical hypoxia' using KCN, an inhibitor of oxidative phosphorylation. KCN was previously reported to decrease the ATP content in hepatocytes (Carini et al., 1995). Under our experimental conditions, endothelial cells were incubated with KCN (1 mM) for 15 min and ATP content as well as neutrophil adherence were assayed. The ATP content of endothelial cells incubated 15 min with 1 mM KCN was 59.3 ± 6.6% ($n = 3$) of the ATP content in control cells and the neutrophil adherence to cells treated with KCN was 3.3-fold higher than the adherence to control endothelial cells. These results suggest that whatever the cause of the decrease in ATP content, a decrease of 40% in ATP content activated endothelial cells and resulted in an increase of the adhesiveness of endothelial cells for neutrophils.

The hypoxia-induced increase in neutrophil adherence to endothelial cells is illustrated in the electron scanning micrographs presented in Fig. 4. The number of neutrophils adherent to the hypoxic endothelial cells is much higher than the number observed on normoxic endothelial cells and the hypoxia-induced adherence was strongly inhibited in the presence of aescine. The number of neutrophils adherent to the endothelium was counted for each set of conditions on several scanning electron micrographs: 0.5 ± 0.7 ($n = 3$); 4.0 ± 1.3 ($n = 12$) and 1.4 ± 1.3 neutrophils ($n = 7$) were, respectively, obtained for normoxia, hypoxia and hypoxia in the presence of aescine at 750 ng/ml. From these values, we calculated an aescine inhibition of 74.3% of the hypoxia-induced neutrophil adherence. These morphological observations support previous data (Arnould et al., 1993) and confirm the results of the biochemical analysis described in Fig. 3.

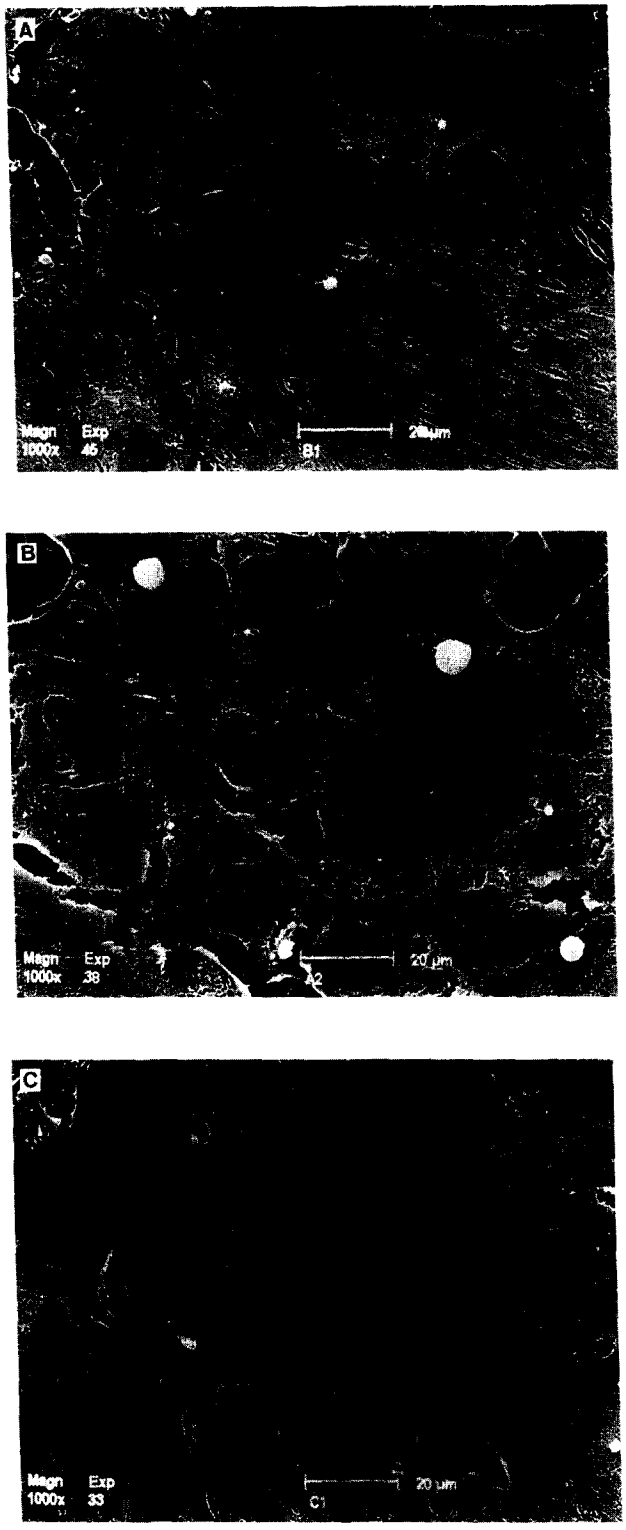

Fig. 4. Scanning electron micrographs of neutrophil adherence to human umbilical vein endothelial cells. Endothelial cells were incubated for 2 h under hypoxia in the presence (C) or in the absence of aescine at 750 ng/ml (B) or maintained in normoxic conditions (A). Magnification = 1000× for all micrographs.

These results show a dose-dependent protection by aescine between 100 and 750 ng/ml from the hypoxia-induced decrease in the ATP content as well as from the activation of phospholipase A_2 and the increase in neutrophil adherence. All these effects were obtained within the same range of concentrations.

4. Discussion

Aescine is widely used in the treatment of chronic venous insufficiency and clinical trials showed that aescine therapy gives significant superior beneficial effects compared to placebo in the treatment of symptoms related to this disease. Besides its effects on capillary filtration, microvascular permeability and edema formation, the exact mechanism of action of this drug is still unclear.

One parameter which could influence the development of chronic venous insufficiency is the trapping of white blood cells occluding the capillaries, and the resulting ischemia which would lead to tissue alterations. Michiels et al. (1994b) recently proposed that the activation of the endothelium by hypoxic conditions occurring during blood stasis could be a possible factor in 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 these events eventually lead to a pathological vessel wall with features similar to the ones observed in varicose vein wall. It had been reported that oxygen levels (tension, saturation and content) in blood from varicose leg veins were found to be significantly lower than those in blood from normal leg veins (McEwan and McArdle, 1971). Measuring the pO_2 under standardized conditions, the authors found that the mean normal leg-vein blood value was around 35 mmHg vs. the mean varicose leg-vein blood value which was around 20–25 mmHg. According to these results, it appears that our hypoxia was more severe than the one observed in the legs of patients with chronic venous insufficiency.

We now showed that aescine was able to inhibit, in a dose-dependent manner, the activation of human umbilical vein endothelial cells by hypoxia as shown by the inhibition of the decrease in the ATP content (EC_{50} = 260 ng/ml), of the activation of phospholipase A_2 (EC_{50} = 90 ng/ml) as well as of the resulting adherence of neutrophils (EC_{50} = 90 ng/ml). The range of inhibition was approximatively the same for all 3 processes. The influence on the ATP level was slightly displaced to higher concentrations. The effects of aescine on the 3 parameters are presented on the same graph for comparison (Fig. 5). These dose-response curves show that aescine protection became significantly different from the lowest concentration tested (1 ng/ml) at 750 ng/ml for ATP content, 100 ng/ml for phospholipase A_2 activity and 100 ng/ml for neutrophil adherence. These results suggest that the drug action is due

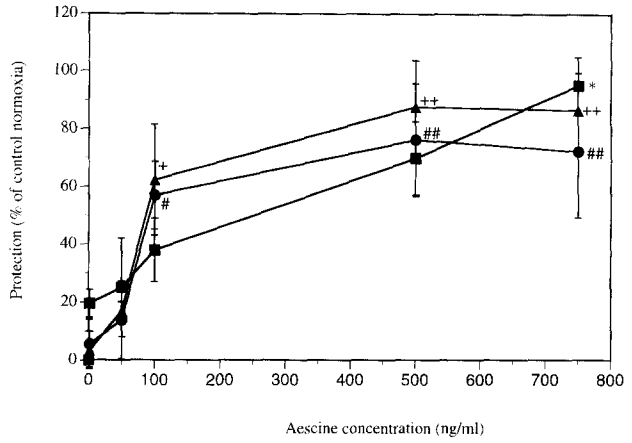

Fig. 5. Comparison of the effect of aescine on the 3 parameters tested in this study: decrease in ATP content (■), activation of phospholipase A_2 (●) and increase in polymorphonuclear neutrophil adherence (▲). The results are expressed as percentages of protection, as means of data from the 3 independent experiments performed in triplicate ($n = 9$) described in Figs. 1–3. * Significantly different from 1 ng/ml of aescine with $P < 0.05$ (■); # or ## significantly different from 1 ng/ml of aescine with $P < 0.05$ or $P < 0.01$ (●); + or ++ significantly different from 1 ng/ml of aescine with $P < 0.05$ or $P < 0.01$ (▲) using an ANOVA-2 with Scheffé's contrasts.

to the inhibition of the initial process which leads to the activation of endothelial cells: the decrease in ATP content.

The fact that aescine prevents the activation cascade induced by the hypoxia which could occur during blood stasis in the leg vein is important because it could explain the inhibition of the infiltration of recruited neutrophils in the underlying tissue; this would then block subsequent local inflammation and the appearance of edema. Aescine would also prevent neutrophil plugging in the microcirculation because the protected endothelium would remain non-adhesive for neutrophils, thus, facilitating the circulation of leukocytes in the blood vessels. This drug was indeed found to be clinically useful to facilitate the microcirculatory flow, which may be beneficial in improving symptoms.

The strong protective effect obtained in this study with aescine on the human endothelial cell activation induced by hypoxia and on neutrophil adherence is perfectly consistent with clinical observations of the preventive effect of the drug. The effect probably represents results from one of the molecular and cellular mechanisms that underlie, among others, protection of the vessel wall. Objective criteria for our understanding of the preventive action of this phlebotonic drug are, thus, provided.

Acknowledgements

We thank the medical and nursing staffs of the Clinique Sainte Elisabeth for providing the umbilical cords. This work was supported by a grant from Fonds de la Recherche

Fondamentale Collective. C. Michiels is a Research Associate of Fonds National de la Recherche Scientifique (Brussels, Belgium). This text presents results of the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. Scientific responsibility is assumed by us. The financial support of Madaus Pharma Belgium is also gratefully acknowledged.

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