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Effects of calycosin on the impairment of barrier function induced by hypoxia in human umbilical vein endothelial cells

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Abstract

The purpose of the present study was to examine the effects of calycosin, an isoflavonoid isolated from *Astragali Radix*, on the impairment of barrier function induced by hypoxia in cultured human umbilical vein endothelial cells. Hypoxia induced an increase in endothelial cell monolayer permeability, indicating endothelial cell barrier impairment. Endothelial barrier dysfunction induced by hypoxia was accompanied by decreases in cytosolic ATP concentration and cAMP level, the development of actin stress fibers and intercellular gap formation, suggesting that the decreases in cytosolic ATP and cAMP levels and rearrangements of F-actin could be associated with an increase in permeability of endothelial monolayers. Application of calycosin inhibited the hypoxia-induced increase in endothelial permeability in a dose-dependent fashion, which is compatible with inhibition of lactate dehydrogenase release, decrease of the fall in ATP and cAMP contents, and improvement of F-actin rearrangements. These findings indicate that calycosin protected endothelial cells from hypoxia-induced barrier impairment by increasing intracellular energetic sources and promoting regeneration of the cAMP level, as well as improving cytoskeleton remodeling.

Keywords: Endothelial cell; Hypoxia; Monolayer permeability; cAMP; ATP; Actin filament

1. Introduction

Ischemia is characterized by in vivo decreases of oxygen delivery and nutriment supply in tissues, which occur in a variety of vascular diseases such as thrombosis, atherosclerosis, myocardial infarction, cerebral ischemia, and venous insufficiency, etc. (Berna et al., 2002; Janssens et al., 2000). Since it composes the main physical barrier localized at the interface between blood and the interstitial fluid of tissues, the endothelium is the first target of ischemia and has been shown to be very sensitive to oxygen tension in the blood; in vivo ischemia in tissues can be thus mimicked by in vitro hypoxia in the endothelium (Janssens et al., 1995). Tissue ischemia disrupts the normal transport pathway for water and solutions (e.g., albumin) across the microvasculature, resulting in vascular leakage and edema. The acute loss of endothelial barrier function under these circumstances is a significant cause of tissue pathology and organ dysfunction, which is accompanied by a decrease of the intracellular cAMP level (Ogawa et al., 1992; Pinsky et al., 1995; Yan et al., 1997), increase of intracellular calcium concentration (Arnould et al., 1992) and rearrangement of cytoskeleton, etc. (Partridge, 1995; Crawford et al., 1996). Resent studies showed that hypoxia strongly activates human umbilical vein endothelial cells. In the primary step, this activation induces a decrease in the ATP content of cells, followed by an increase in cytosolic calcium concentration, and subsequently activates phospholipase A_2 that then synthesizes a large amount of prostaglandins and platelet-activating factor. Hypoxic tissue can thus initiate an inflammatory response via activation of endothelium, which will then induce tissue injury (Michiels et al., 2000; Berna et al., 2002). Many investigations have demonstrated that some compounds, especially flavonoids, isolated from plant medicines such as escin, hydroxythylrutoside, Daflon, diosmin, hesperidin, bilobalide, were able to inhibit endothelial hyperpermeability or to prevent its activation by hypoxia (Frick, 2000; Bouaziz et al., 1999; Bouskela and Donyo, 1997), and then to exert beneficial

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pharmacological effects on endothelial dysfunction and vascular disease.

In China, *Astragali Radix*, the dry root of *Astragalus membranceus* (Fisch) Bge, has been used for centuries as an important medicine to reinforce vital energy, to strengthen superficial resistance, and to promote the discharge of pus and the growth of new tissue (Lin et al., 2000). It contains numerous triterpene saponins, polysaccharides, and flavonoids, which are known to have various biological effects, such as improving immune function (Wang et al., 2002), inhibiting the increase of microvascular permeability induced by histamine (Wu and Hu, 2001), anti-inflammatory (Zhang et al., 1984), antitumor (Kim et al., 1998), antiproliferation (Kao et al., 2001), and anti-oxidation (Hong et al., 1994; Toda and Shirataki, 1998). Calycosin, an isoflavonoid, is the major active component in *Astragali Radix*. Recently, it has been chosen as a marker substance for the chemical evaluation or standardization of *Astragali Radix* and its products (Lin et al., 2000). Calycosin also displays beneficial effects such as antitumor (Kim et al., 1998), antioxidation (Toda and Shirataki, 1998), anti-ischemia (unpublished experimental results) and inhibition of hyperpermeability induced by low osmolarity (Wu et al., 2000). However, the effect of calycosin on endothelial barrier function during hypoxia remains unclear. The aim of this study was to examine the actions of calycosin on the impairment of barrier function induced by hypoxia in human umbilical vein endothelial cells and to investigate the underlying mechanism(s). The results demonstrate that calycosin protected endothelial cells from hypoxia-induced barrier dysfunction and its effect was accompanied by restoration of the diminished intracellular ATP and cAMP concentration, inhibition of lactate dehydrogenase (LDH) release, and improving of cytoskeletal rearrangement.

2. Materials and methods

2.1. Materials and agents

Chemically pure calycosin was obtained from the chemistry laboratory, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine (Shanghai, P.R. China). It was identified on the basis of chemical and spectroscopic evidence. The chemical structure of calycosin is shown in Fig. 1. Transwell polycarbonate membranes

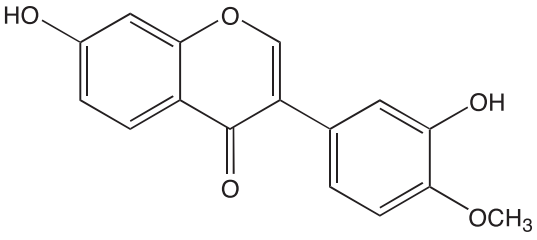

Fig. 1. Chemical structure of calycosin (molecular weight = 284).

were obtained from Costar (Cambridge, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and L-glutamine were purchased from Gibco (Grand Island, NY, USA). Fluoresceinisothiocyanate-dextran (FITC-dextran) and FITC-phalloidin were obtained from Molecular Probe (Leiden, Netherlands). Other biochemical reagents were from Sigma (St. Louis, MO, USA) or Amresco (Solon, OH, USA).

2.2. Endothelial cell culture and treatment

Human umbilical vein endothelial cells were isolated, cultured and characterized with von Willebrand factor, as we described previously (Fan et al., 2002). The endothelial cells were cultured in gelatin-coated culture flasks to achieve confluence in DMEM supplement with 2 mM L-glutamine, 50 μg/ml endothelial cell growth supplement (Sigma), 20 mM HEPES, 10% fetal bovine serum, 5 U/ml heparin, 100 IU/ml penicillin and 100 μg/ml streptomycin. Confluent cells were used between the second and the sixth passages. Cell treatment with calycosin was conduced as follows: calycosin in fresh culture medium was added and incubated with endothelial cells for 30 min before hypoxia, and then incubated further during the hypoxic procedure. Meanwhile, blank cells subjected to normoxia or to hypoxia alone were regarded as "control" or "hypoxia", respectively.

2.3. In vitro hypoxia

The confluent endothelial monolayer was washed out with DMEM and then was placed in a sealed Perspex chamber filled with 98% N_2 and 2% CO_2 moist atmosphere at 37 °C. The oxygen content in the chamber was reduced to a constant concentration, 0.5% vol/vol. The medium was reduced to a uniform thin layer to decrease the diffusion distances of atmospheric gases. The concentration of oxygen after various periods of hypoxia in the culture-incubated medium was monitored with a digital oxygen meter.

2.4. Determination of monolayer permeability

Endothelial cells were grown to achieve confluence on 12-well plate Transwell filters with 0.4 mm pore size (Partridge, 1995). To avoid quenching of fluorescence during determinations, endothelial cells were cultured in phenol red-free DMEM medium containing 2% fetal bovine serum. Fluoresceinisothiocyanate-dextran, FITC-dextran, 50 μM with molecular weights of both 4400 and 77,000 was introduced in the luminal chamber. The wells were placed in a 37 °C bath and 100 μl samples were removed from the abluminal chamber at 30-min intervals for 8 h. This volume was immediately replaced by DMEM to prevent hydrostatic pressure changes. Fluorescence in the abluminal samples was measured by a F4500 fluorimeter (Hitachi, Tokyo, Japan) and compared with a standard curve of fluorescence made with various dilutions of the FITC-dextran. The data

from each well were used to construct a linear regression curve relating time and FITC-dextran concentration. The rate of flux across was taken from the slope of this regression (Siflinger-Birnboim et al., 1987). Data from the wells producing linear regression with a correlation coefficient of less than 0.9 were discarded.

2.5. Measurements of cAMP

Endothelial monolayers seeded on 60-mm tissue culture dishes were exposed to the appropriate experimental conditions. After incubation, cytosolic cAMP content was determined by enzyme-linked immunosorbent assay (ELISA) (Talor et al., 1998). Briefly, cAMP was extracted from washed monolayers with extraction buffer (10% trichloroacetic acid containing 5 mM phosphodiesterase inhibitor isobutylmethylxanthine, Sigma) at 4 °C, and then cells were scraped and centrifuged at 10,000 × *g* for 5 min to remove debris. Trichloroacetic acid was removed by extracting three times with water-saturated diethyl ether which was removed by heating to 70 °C for 5 min. Samples were diluted with assay buffer, and cAMP was quantified using an ELISA assay kit (Cayman, Ann Arbor, MI, USA) and total protein was measured using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA) both used according to the manufacturer's instructions. The data were converted to concentration using a daily standard curve, and final results were expressed as pmol cAMP per mg of total protein.

2.6. Measurements of ATP

After exposure to the appropriate experimental conditions, endothelial cell monolayers were rinsed three times with chilled phosphate-buffered saline (PBS, pH 7.4) and scraped into cold 0.04 M Tris-borate buffer (pH 9.2). To release intracellular ATP, the cell suspension was placed immediately into a boiling water bath for 5 min and then cooled on ice (Lee and Fanburg, 1987). Centrifugation was at 12,000 × *g* for 10 min at 4 °C. ATP was quantified using the luciferin-luciferase method with an assay kit (Molecular Probe, Leiden, Netherlands), and luminescence was recorded at 560 nm on a F4500 Luminspectrometer (Hitachi, Tokyo, Japan). The concentration of ATP was calculated from a daily standard curve and corrected for the total protein concentration. To assess stability of ATP during the isolation procedure, experiments were carried out in which ATP at a known concentration was added to the cell suspension prior to extraction of cells. ATP recovery by this method was 98.4%.

2.7. Measurements of LDH

LDH measurement: Cells were subcultured in 24-well plates to achieve confluence. Before hypoxia, the medium was replaced by 300 μl phenol red-free DMEM. The release of lactate dehydrogenase (LDH) into the medium by endothelial cells was determined using a method previously described (Decker and Lohmann-Matthes, 1988). Results for LDH release were expressed as percentages of the maximum LDH present in the culture (releasing + intracellular), as reported previously (Gobbel et al., 1994).

2.8. Measurement of actin filament by fluorescence studies

Staining of endothelial cytoskeletal F-actin was achieved using FITC-phalloidin (Wulf et al., 1979). The control or the calycosin-treated endothelial cells on gelatin-coated glass coverslips (Bland, Germany) were fixed in 3.7% formalin, permeabilized in 0.5% Triton X-100, and incubated with 50 μg/ml FITC-phalloidin for 40 min at room temperature in a humid atmosphere. After three washes, the coverslips were mounted with 80% glycerol in PBS. Fluorescence was examined with an Axioskop-2 fluorescent microscope (Zeiss, Germany) equipped with an ISIS system cool-CCD (Zeiss).

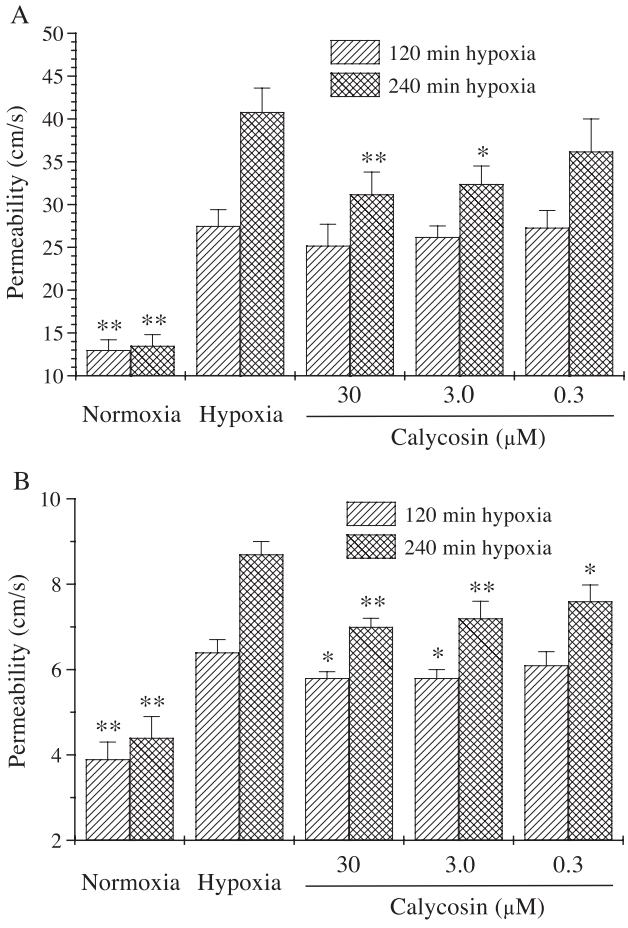

Fig. 2. Effect of calycosin on macromolecular permeability across endothelial monolayers exposed to hypoxia for 120 and 240 min. The endothelial monolayers were incubated in a normoxic or hypoxic atmosphere in the absence or presence of 0.3–30 μM calycosin. The macromolecular permeability was measured with FITC-dextran (A: molecular weight of 4400; B: molecular weight of 77,000). Results are means ± S.D. $*P<0.05$ and $**P<0.01$, compared with hypoxia ($n=6$).

The changes in the elements of F-actin was quantitated as previously described (Goeckeler and Wysolmerski, 1995). In brief, monolayers were fixed with 3% formaldehyde, permeabilized with 0.5% Triton X-100, stained with 200 μg/ml FITC-phalloidin, and extracted with methanol. After the extracted methanol was centrifuged at 10,000 × *g* for 5 min, FITC-phalloidin in the supernatant was measured with a F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) set to the following: excitation 496 nm, emission 516 nm. Quantitated F-actin corrected for total protein content was expressed as a percentage of the control.

2.9. Statistical analysis

All data were expressed as the means ± S.D. Statistical significance was assessed by Student's *t*-test, and a *P* value of less than 0.05 accepted as a significant difference.

3. Results

3.1. Effect of calycosin on macromolecular permeability across human endothelial monolayers

The assay for permeability across endothelial monolayers was described previously (Hashida et al., 1986), and is based on the transporting flux of FITC-dextran through the intercellular spaces of the endothelial junctions. In present study, FITC-dextran with molecular weight of 4400 or 77,000 was used as a probe of macromolecular permeability across endothelial monolayers. Exposure of the monolayers with hypoxia for 120 and 240 min resulted in increased permeability to FITC-dextran (Fig. 2A and B). Treatment with the various concentrations of calycosin during 240 min of hypoxia significantly attenuated the increased permeability to FITC-dextran in a dose-dependent fashion, whereas the effect of calycosin on 120-min hypoxia-induced hyperpermeability was relatively mild and only observed in the permeability assay with FITC-dextran with molecular weight of 77,000.

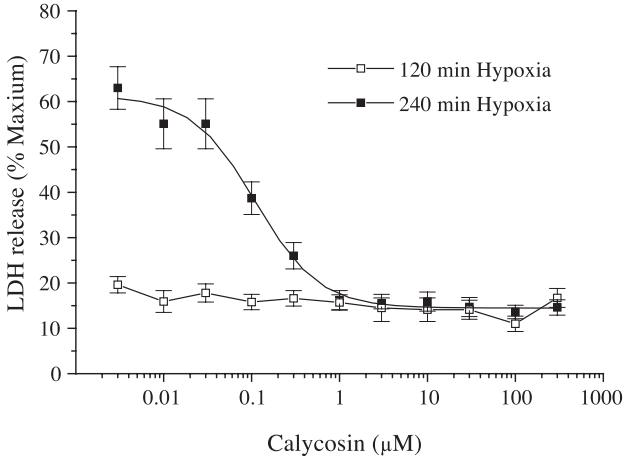

Fig. 3. The effect of calycosin on the release of lactate dehydrogenase (LDH) from endothelial cells exposed to hypoxia for 120 and 240 min. Hypoxia for 240 min (■), but not for 120 min (□), dramatically increased the release of LDH, and treatment with calycosin significantly attenuated LDH release during 240-min hypoxia with an IC_{50} of 0.11 ± 0.02 μM. Results are means ± S.D. (n = 10–12).

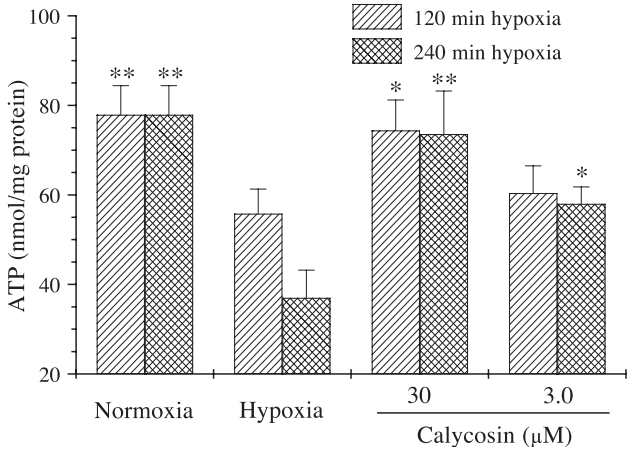

Fig. 4. Effect of calycosin on intracellular ATP concentration in endothelial cells exposed to hypoxia for 120 and 240 min. The endothelial monolayers were incubated in a normoxic or hypoxic atmosphere in the absence or presence of 0.3–30 μM calycosin. Results are means ± S.D. *P < 0.05 and **P < 0.01, compared with hypoxia (n = 6).

3.2. Effect of calycosin on LDH release

Treatment of endothelial cells with 30 μM calycosin for 240 min failed to induce a significant LDH release (11.5% ± 2.4%, compared with the normoxic control 12.3% ± 2.1%, P>0.1), which implies that there is no cytotoxic effect of calycosin on human endothelial cells at the dose used. Compared with the normoxic situation, 120 min of hypoxia

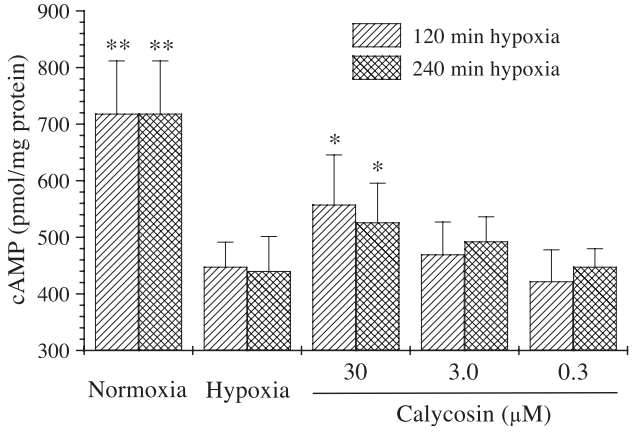

Fig. 5. Effect of calycosin on intracellular cAMP content in endothelial cells exposed to hypoxia for 120 min and 240 min. The endothelial monolayers were incubated in a normoxic or hypoxic atmosphere in the absence or presence of 0.3–30 μM calycosin. Results are means ± S.D. *P < 0.05 and **P < 0.01, compared with hypoxia (n = 5–8).

did not induce a significant release of LDH (16.1% ± 3.2%). However, 240 min of hypoxia dramatically increased the release of LDH (62.1% ± 5.9%). The effect of calycosin on the release of LDH from endothelial cells exposed to hypoxia is shown in Fig. 3. Treatment with the various concentrations of calycosin during 240-min hypoxia significantly attenuated the release of LDH from endothelial cells. The calculated IC_{50} value for calycosin inhibiting the release of LDH was 0.11 ± 0.02 μM. On the basis of the relationships between release of lactate dehydrogenase and permeability across human endothelial monolayers during hypoxia, we considered that endothelial cell damage may not be responsible for the hypoxia-induced permeability increase.

3.3. Effect of calycosin on cytosolic ATP and cAMP contents

In order to investigate the effect of calycosin on the energy depletion during hypoxia, we assayed the intracellular level of ATP in endothelial cells. As shown in Fig. 4, the control value in resting cells was 77.9 ± 6.5 nmol/mg protein ($n = 6$). Exposure of endothelial cells to hypoxic conditions caused a significant decrease in intracellular ATP contents with the following time course: after 120-min hypoxia, it decreased by about 28% ($n = 6$), and hypoxic treatment for 240 min resulted in approximately 52% loss of intracellular ATP compared with the normoxic cells, reach-

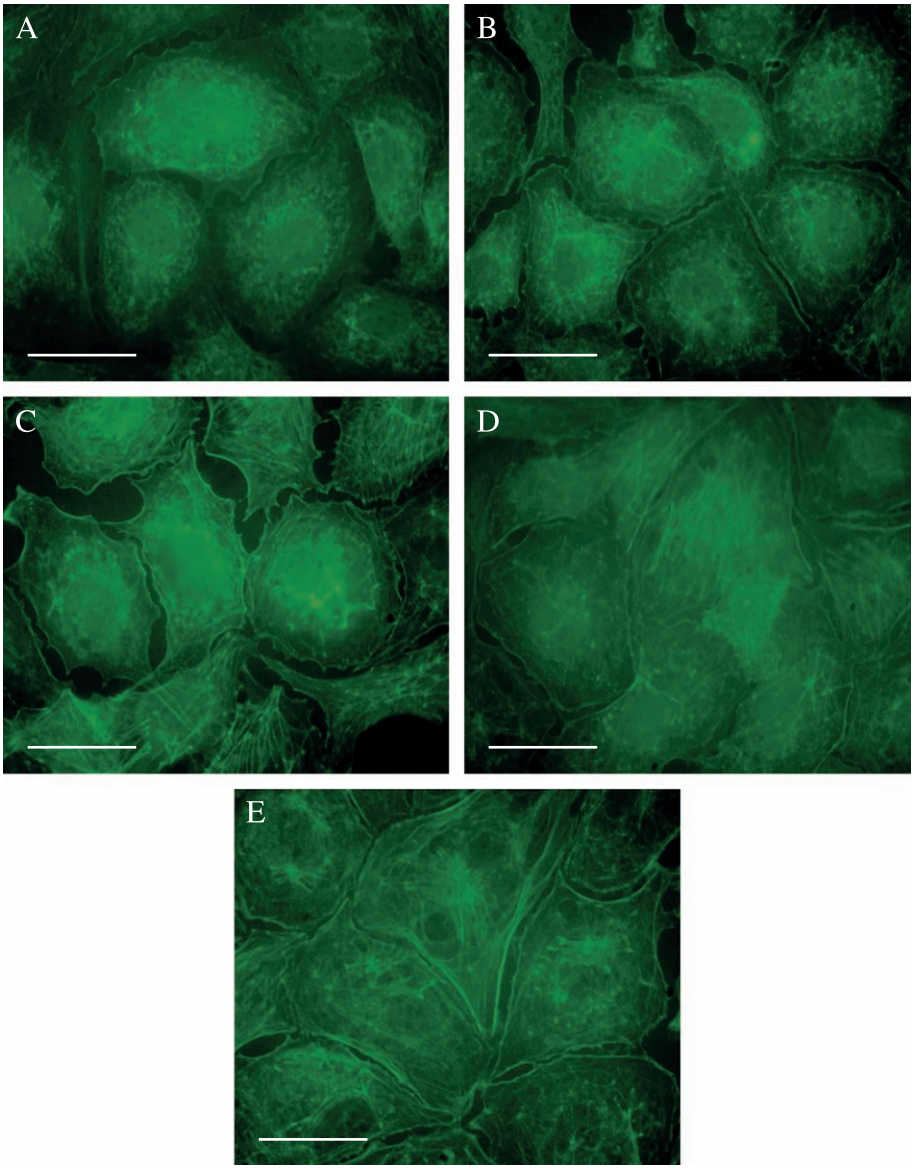

Fig. 6. Effect of calycosin on actin cytoskeleton remodeling in endothelial cells exposed to hypoxia for 120 and 240 min. F-actin in the endothelial cells exposed to hypoxia was studied by fluorescence microscopy using FITC-phalloidin as the probe. (A) Actin cytoskeleton in normoxic cells as control, (B) the endothelial cells exposed to hypoxia for 120 min, (C) the endothelial cells exposed to hypoxia for 240 min, (D) the endothelial cells exposed to hypoxia for 120 min with 30 μM calycosin pretreatment, (E) the endothelial cells exposed to hypoxia for 240 min with 30 μM calycosin pretreatment. Representative figures were taken from four independent assays (Bar: 25 μm).

ing 37.0 ± 6.2 nmol/mg protein ($n = 6$). It is of note that treatment with calycosin at the concentration of 3.0 μM restored the intracellular ATP contents to 60.4 ± 6.1 and 58.8 ± 9.3 nmol/mg protein, and calycosin at 30 μM increased the intracellular ATP contents to 74.4 ± 6.8 and 73.6 ± 9.6 nmol/mg protein (hypoxia for 120 and 240 min, respectively). These results indicate that 30 μM calycosin almost completely inhibited the decrease of intracellular ATP level induced by hypoxia.

We also studied the effect of calycosin on the cAMP level in cultured endothelial cells during hypoxia. As shown in Fig. 5, the intracellular cAMP level under normoxic conditions was 718.5 ± 93.2 pmol/mg protein ($n = 8$). After endothelial cells were exposed to hypoxia for 120 and 240 min, the intracellular cAMP level was decreased to 447.8 ± 43.6 and 440.4 ± 61.0 pmol/mg protein, respectively. Application of calycosin at the concentrations of 0.3, 3.0 and 30 μM inhibited the decreases of cAMP contents of the endothelial monolayers induced by hypoxia in a dose-dependent manner ($n = 5–8$).

3.4. Effect of calycosin on actin filament

The permeability increase on contraction of endothelial cells in hypoxia is reflected by the changes in the actin cytoskeleton. Fig. 6 illustrates immunofluorescent staining of F-actin with FITC-phalloidin. Under normoxic conditions, endothelial cells formed a confluent monolayer in which the F-actin was distributed primarily in a cortical ring with a few stress fibers spanning the cells. After hypoxia for 120 and 240 min, the endothelial cells displayed dissolution of cortical rings. These cytoskeletal rearrangements show cell contraction and led to intercellular gap formation, which is compatible with increased permeability during the same hypoxic periods. The cytoskeletal rearrangement in hypoxia was partly but efficiently prevented by pretreatment with calycosin at 30 μM, as indicated by diminished cell retraction (Fig. 6). Moreover, quantitative F-actin measurement indicates that 240 min of the hypoxia decreased F-actin content in endothelial cells by about 15% compared with the normoxic cells, and that pretreatment with calycosin at 3.0 and 30 μM inhibited the attenuation of endothelial F-actin content by about 3% and 13%, respectively (Fig. 7).

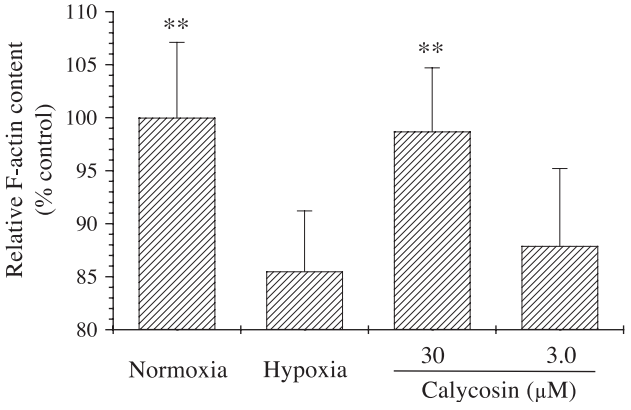

Fig. 7. Effect of calycosin on relative F-actin content in endothelial cells exposed to hypoxia for 240 min. The endothelial monolayers were incubated in a normoxic or hypoxic atmosphere with or without 30 μM calycosin. After treatment, the endothelial monolayers were stained with FITC-phalloidin and extracted with methanol. The extracted fluorescence was measured and data are expressed as relative F-actin content (Goeckeler et al., 1995). Results are means ± S.D. *$P < 0.05$ and **$P < 0.01$ compared with hypoxia ($n = 6–8$).

4. Discussion

Endothelium plays a key role in many physiological responses such as regulation of vascular tone, permeability, homeostasis, angiogenesis, and synthesis of biologically active factors. Due to its location at the luminal vascular surface, endothelium must be able to respond to a wide range of environmental alterations. One of these is the hypoxic conditions that is usually associated with many vascular disorders, especially those initiated by ischemia (Janssens et al., 1995). The first step of pathological activation in endothelial cells by hypoxia in vitro is the decrease of intracellular ATP content. During hypoxia, glycolytic metabolism is enhanced in order to compensate for the decreased activity of the mitochondrial oxidative phosphorylation resulting from oxygen lack. However, glycolysis is not sufficient to regenerate fully the ATP needed by the cells during hypoxia, and consequently intracellular ATP concentration decreases. This leads to a series of modifications in cellular functions: lowering or arrest of the Na^+ and Ca^{2+} ATPase pumps, alteration of the plasma membrane and of the regulation of cellular volume, dissociation of the cytoskeleton elements, inhibition of protein synthesis, mitochondria swelling and lysosome dilation and rupture (Michiels et al., 1993). In the present study, we showed that calycosin is able to inhibit the decrease of ATP concentration in endothelium during hypoxia in a dose-dependent manner, suggesting that calycosin exerts its beneficial effect by maintaining a high energy level in human endothelial cells during hypoxia. A decrease in intracellular ATP caused by hypoxia could be implicated in the underlying mechanism by which this hypoxia increases permeability across the endothelial monolayers for macromolecules. Since it is well known that the integrity of tight junctions between adjacent cells is regulated by tyrosine phosphorylation and is ATP-dependent, a significant decrease of the ATP level in the endothelial monolayers could explain the disorganization of tight junctions, the rearrangement of the F-actin, and the appearance of gaps between the cells leading to an increase in the permeability. In contrast, the study of the endothelial permeability in porcine pulmonary artery confirmed that reduction of endothelial cell ATP levels with the metabolic inhibitors did not alter the flux of albumin or dextran across the endothelial monolayers, thus ATP depletion, by itself, does not explain

oxidant-induced changes in endothelial permeability to macromolecules (Wilson et al., 1990). The present work showed that the reduction in ATP levels appears to be one of the main mechanisms involved in the increases of permeability in endothelial monolayers during hypoxia. This conclusion is based on the marked linkage observed between the improvement of permeability and recovery of ATP level on treatment with calycosin during hypoxia for120 and 240 min.

Many previous studies have demonstrated that the modification of transport of macromolecules across the endothelial monolayers was associated with a decrease of the intracellular cAMP level. During hypoxia, there is a close relationship between increasing permeability to a trace solute and diminution of the intracellular cAMP content. A decreased cAMP in hypoxic endothelium reflected specific redirection of cAMP metabolism, and resulted from an enhanced breakdown or extrusion of cyclic nucleotide into the medium. cAMP has been shown to be an important determinant of endothelial barrier function, and agents which elevate the cAMP level, in vitro and in vivo, could diminish vascular permeability (Farrukh et al., 1987; Stelzner et al., 1989). The decrease in cAMP content might be central in the hyperpermeability consequent to hypoxia: supplementation of intracellular cAMP with membrane permeable cAMP analogs enhances barrier function, since addition of pertussis toxin, which diminished the hypoxia-induced fall in cAMP, decreased vascular leakage; and agents which induced sustained elevation of cAMP, such as dexamethasone, improved barrier function (Pinsky et al., 1995; Adkins et al., 1992). Our data indicate that the decline of the intracellular cAMP content was detected after endothelial hypoxia for 120 and 240 min. The presence of calycosin attenuated the hypoxia-induced decrease of cAMP level, suggesting that the effects of calycosin on the increase of permeability to macromolecules in endothelial monolayers resulted from restoration of the cAMP level, at least partly. It is also possible that cAMP exerts a protective effect on barrier function, an effect which is related to decreasing intercellular junctional size. The results presented here provide direct evidence that calycosin acts on endothelial monolayers to protect them from hypoxia-mediated increases of macromolecule permeability due to changes in cytoskeletal distribution of F-actin. In addition, calycosin induced a significant increase in quantitative F-actin elements in endothelial cells compared to those in hypoxic cells. These findings suggest that preincubation with calycosin prevents hypoxia-mediated rearrangements of F-actin and support the possibility that calycosin, directly or indirectly, stabilizes the endothelial cell cytoskeleton and intercellular junction thus maintaining the function of the endothelial barrier.

In summary, the present study confirmed that hypoxia induced an increase in endothelial cell monolayer permeability and that this was prevented by the application of calycosin. The results indicate that calycosin could inhibit hypoxia-induced hyperpermeability, an effect which was accompanied by diminishing of the hypoxia-induced cell damage and falls in cAMP and ATP, and the rearrangement of cytoskeleton, which suggests that calycosin could be a promising prodrug for hypoxia-related vascular disease therapy.

Acknowledgements

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