Abstract. Background: Erianin is a natural product derived from Dendrobium chrysotoxum, with promising antitumor activity. Materials and Methods: To evaluate the metabolic effect of erianin, a cytosensor assay for acidification rate, MTT assay, measurement of lactate, glucose and ATP were performed in human umbilical vein endothelial cells (HUVECs) exposed to 1-100 nM erianin. JNK/SAPK activity was detected by Western blot. Results: Twelve- or 24-hour incubation with erianin induced a dose-dependent metabolic inhibition, as indicated by reduced acidification rate and cell viability, with an endothelium-selectivity. Erianin caused decreases in lactate production, glucose consumption and intracellular ATP level. Pretreatment with the JNK/SAPK inhibitor SP600125 significantly abolished these inhibitory responses, and especially restored the erianin-induced decreases in ATP and the erianin-induced phosphorylation of JNK/SAPK with dose- and time-dependence. Conclusion: Erianin inhibited endothelial metabolism in a JNK/SAPK-dependent manner. This mechanism may be involved in the potential antitumor and antiangiogenic actions of erianin.

2-Methoxy-5-[2-(3,4,5-trimethoxy-phenyl)-ethyl]-phenol, erianin, is a low molecular weight natural product isolated from Dendrobium chrysotoxum Lindl. (1), which is often used as an antipyretic and an analgesic in traditional Chinese medicine. Structurally, erianin contains two phenyl rings linked by a two carbon bridge with several methoxyl substitutions on the phenyl rings and belongs to the bibenzyl derivatives, among which many compounds have shown antiviral (2), antibacterial (3), or antiprostatic activities (4). Structurally similar to erianin, some stilbene (5) and phenanthrene derivatives (6) were found to display potent antitumor activity; typically, combretastatin A-4 has been a promising candidate for antitumor therapy to control the aberrant angiogenesis occurring in tumor development (7).

Previous studies have demonstrated the antitumor activity of erianin in vivo and in vitro (8,9). Moreover, experimental results indicated that erianin could inhibit the proliferation of cultured human promyelocytic leukemia HL-60 cells and cause cell apoptosis (9), and it could reverse multidrug resistance in B16/h MDR-1 cells (10). Furthermore, our findings indicated that erianin at a concentration as low as 10 nM displayed a potent antiangiogenic activity in vivo and in vitro (submitted for publication, Gong YQ et al.), in consistence with the experimental data of the above mentioned vascular-target agent combretastatin A-4 (7). However, the metabolic effect of erianin on the endothelium and the underlying cellular or molecular mechanisms remain unclear.

The present study aimed at elucidating the mechanism of the pharmacological effects of erianin on human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Reagent and cell lines. Erianin, isolated from Dendrobium chrysotoxum Lindl. (1), identified by IR, UV, NMR and MS, with a purity of 98%, was dissolved in DMSO as a 3 mM stock solution. BAPTA-AM, calphostin C, endothelial cell growth supplements (ECGS), 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) and PD98059 were obtained from Sigma (St. Louis, MO, USA). H89 and SP600125 were purchased from Biomol (Plymouth Meeting, PA, USA) and SB203580 was from Calbiochem (Beverly, MA, USA). The rabbit polyclonal antibodies to phospho-JNK/SAPK and total JNK/SAPK were from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated secondary antibodies were from Rockland (Gilbertsville, PA, USA). Mouse fibroblasts NIH/3T3, human melanoma A375 and hepatoma Bel7402 cells were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, P.R. China). Unless otherwise mentioned, reagents for cell culture were purchased...
Cells culture and treatment. Human umbilical vein endothelial cells (HUVECs) were isolated, cultured and characterized with von Willebrand factor, as we described previously (11,12). HUVECs were cultured in gelatin-coated culture flasks to achieve confluence at 5% CO2-95% air moist atmosphere in DMEM medium supplemented with 2 mM L-glutamine, 20 mM HEPES, 10% FBS, 50 µg/ml ECGS, 5 U/ml heparin, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C. HUVECs were used between the second and the sixth passages. NIH/3T3, A375 and Bel7402 cells were cultured in the same medium but without ECGS or heparin. Cells were pretreated with various signal regulators or medium alone for 1 hour and then exposed to erianin. For measurement of lactate and glucose, Krebs-Henseleit buffer (K-H buffer) was used as medium, the components of which were: 5.5 mM glucose, 1.3 mM CaCl2, 1.3 mM MgCl2, 124 mM NaCl, 3.5 mM KCl, 1.25 mM KH2PO4, 26.3 mM NaHCO3, pH 7.4, following bubbling with 5% CO2 in air.

Microphysiometer measurement. Extracellular acidification rates were evaluated as an index of metabolic activity by the application of Cytosensor Microphysiometer (Molecular Devices, Sunnyvale, CA, USA), as we described previously with minor modification (11). Briefly, cells were subcultured and allowed to attach onto the polycarbonate membrane (3-µm pore size) at a density of 5 x 10⁵ cells per transwell in DMEM supplemented with 10% FBS for 24 hours before experiment. On the day of experiment, the assembled units, including transwells with treated cells, spacer rings and insert cups, were transferred into the microphysiometer chambers. Low-buffered DMEM (with no HEPES or bicarbonate, but with 11 ml of 4M NaCl per 1L DMEM, to preserve the osmotic balance) in the presence or absence of 10 nM erianin was used as running media with a perfusion rate of 100 µl/min at 37°C. The extracellular acidification rate was measured as a change in pH over time for 12 hours, which was determined by 30-s potentiometric rate measurements (1 µV/s; pump off cycle) after an 80-s pump cycle with a 10-s delay (120-s total cycle time). Since the acidification rate data were dependent on cell seeding density and uniformity, acidification rates were normalized as percentage of the baseline.

Lactate assay. The production of lactate by HUVECs exposed to erianin was determined using a commercially available lactate assay kit (Sigma). The amount of lactate was measured in medium as previously described with minor modifications (13). Briefly, HUVECs at 80% confluence in 6-well plate were washed twice with 600 µl K-H buffer and exposed to erianin for various time-intervals with or without 1-hour pretreatment of signal regulators. Twenty µl of the medium was collected, centrifuged at 200 x g for 10 minutes and kept at 4°C. The measurement was performed using lactate dehydrogenase (LDH) and NAD+ according to the manufacturer’s protocol. The lactate levels were measured as absorbance at 340 nm by using a SPECTRAnax 390 microplate reader (Molecular Devices). With a daily standard curve, values were transformed to mmol/L.

Glucose measurement. The glucose consumption of HUVECs was evaluated by measurement of the medium glucose levels using a glucose assay kit (Sigma). HUVECs in a 6-well plate were rinsed twice with K-H buffer and subjected to appropriate treatment. During incubation with 600 µl K-H buffer in the presence or absence of erianin, 10 µl medium were withdrawn at different time-points. After 10 minutes centrifugation at 200 x g, the level of glucose in the supernatant was determined by a hexokinase/glucose-6-phosphate dehydrogenase method according to the manufacturer’s manual. The absorbance at 340 nm was determined and data were expressed as mmol/L using a daily standard curve.

Cell viability assay. Cell viability was determined by use of a standard MITT assay (14). Erianin was dissolved in DMSO. The DMSO concentration in the medium was less than 0.1% (v/v). Cell viability was evaluated following a continuous 12- or 24-hour exposure to a range of drug concentrations on HUVECs, NIH/3T3, A375 and Bel7402 cells in 96-well plate. The absorbance at 540 nm was determined by using the SPECTRAnax 390 microplate reader. Each sample was assayed in 8-duplicates and each assay was repeated at least four times. The experimental data was normalized for comparison.

ATP measurement. Intracellular ATP was determined using a luciferin/luciferase assay (13,15) with a bioluminescent FL-ASC kit (Sigma). In brief, after washing and treating, HUVECs in 24-well plate were lysed with 200 µl somatic cell ATP releasing reagent (Sigma). The supernatants collected from 10 minutes centrifugation at 1,000 x g were mixed with assay mix (FL-AAAM) and the light emission at 560 nm was measured with a F4500 luminometer (Hitachi, Tokyo, Japan). The values were calculated based on a daily ATP standard curve and corrected with data of protein concentration obtained from a standard biocinchenic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

Western blotting. Treated HUVECs were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM diethiothreitol, 0.5 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM NaF, 100 µg/ml PMSF, 1 µg/ml aprotinin, 2 µg/ml leupeptin, pH 7.5). Protein concentration was determined by the BCA protein assay. Twenty µg of total protein were suspended in loading buffer (187.5 mM Tris-HCl, 6% SDS, 30% glycerol, 150 mM diethiothreitol, 0.1% bромophenol blue, pH 6.8), heated to 95°C for 10 minutes and analyzed in 10% SDS-PAGE. Following transfer of protein to nitrocellulose membranes (Pharmacia, MA, USA), the membrane was placed for 2 hours in blocking buffer (PBS containing 5% milk and 0.05% Tween-20), blocked overnight in blocking buffer at 4°C with the primary antibody (anti-phospho-JNK/SAPK, dilution of 1:2,000), washed three times for 15 minutes in wash buffer (0.05% Tween-20 in PBS) and then incubated for 1 hour at room temperature with HRP-conjugated secondary antibody (dilution of 1:5,000). Blots were washed in wash buffer three times for 15 minutes. Proteins were detected by SuperSignal Chemiluminescent Substrate (Pierce) on X-ray films (Fujifilm, Tokyo, Japan). The membranes were stripped using the re-blot buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) at 50°C for 20 minutes, and re-immunoblotted/detected with an antibody to total JNK/SAPK as above.

Statistics. All data were expressed as the means±SEM. Statistical significance was assessed by a Student’s t-test with computer software SigmaStat 2.0 (SPSS, Chicago, IL, USA) and a p-value of less than 0.05 was accepted as a significant difference.

Results

In the present study, experimental data indicated that erianin induced a metabolic inhibition in HUVECs as shown by...
measurement of extracellular acidification rate (Figure 1). After transient stimulation within the first 60 minutes, erianin at different doses ranging from 1 to 100 nM produced a sustained inhibition of the acidification rate, an index of cell metabolism (16). This inhibitory response was dose-dependent during the first 12-hour incubation of HUVECs (Figure 1) and maintained for 24 hours (data not shown). In addition, this metabolic inhibition was restricted to endothelial cells, with very limited and insignificant inhibition of other cells, including fibroblasts NIH/3T3 (98.3%±2.5% of the control area under the acidification rate curve, n=4), melanoma A357 (97.3%±2.4% of control, n=5), and hepatoma Bel7402 cells (97.1%±2.7% of control, n=5), exposed to 10 nM erianin for 12 hours by acidification rate assay. However, an efficient suppression in HUVECs (89.1%±1.1% of control, n=5) was observed. Moreover, similar data was obtained from the MTT assay after 12- or 24-hour exposure to 10 nM erianin (data not shown).

Furthermore, cytotoxicity assay was performed in erianin-exposed HUVECs, as shown by a LDH release assay previously described (12), reflecting the destruction of plasma membrane occurring in cell death. During 12 hours of incubation, erianin (1 to 100 nM) was found to be noncytotoxic, since it failed to cause cell death. After 24 hours, erianin at 100 nM induced cytotoxicity to HUVECs (data not shown).

To evaluate the effect of erianin on glycolysis, lactate production and glucose consumption were respectively assayed in HUVECs exposed to erianin. In the short (2-hour) erianin exposure lactate secretion was transiently accelerated, however, further incubation produced a slight and sustained decrease in lactate production, with 85.7%±5.8% of control for 10 nM erianin and 75.6% for 100 nM after 12 hours (Figure 2A). After 24 hours, further inhibition (69.5%±4.3%) was induced by 10 nM erianin, but the level of lactate after treatment with 100 nM erianin returned to 90.6%±4.4%, which might be due to leakage of lactate from dying or already dead cells (24-hour incubation with 100 nM erianin produced cytotoxicity in HUVECs). On the other hand, during 24 hours of erianin treatment, the glucose consumption in HUVECs, measured as medium glucose concentration, was affected dose-dependently (Figure 2B). In consistency with the findings of lactate production, after a transient acceleration (2 hours), glucose consumption was partly but significantly shut off, while the control cells continued to consume glucose for 24 hours.

To investigate what signal pathways were involved in these endothelial metabolic inhibitions induced by erianin, the agents that modulated the key cellular transduction signaling of metabolic events were applied: H89 as a PKA inhibitor, calphostin C as a PKC inhibitor, PD98059 as a ERK/MAPK inhibitor, SP600125 as a JNK/SAPK inhibitor, SB203580 as a p38/MAPK inhibitor and BAPTA-AM as a cell membrane-permeable calcium chelator. As shown in Figure 3A, 1 hour
of pretreatment with SP600125, but not calphostin C, efficiently attenuated 10 nM erianin-induced inhibition of acidification rate in HUVECs. A summary of the effects of these signaling regulators on the inhibitory responses of acidification rate induced by erianin is given in Figure 3B, indicating that H89, SB203580 and BAPTA-AM all failed to induce a similar suppressive effect as SP600125, whereas PD98059 further sensitized the acidification rate response induced by erianin. Similar data were also given from the cell viability/MTT assay (Figure 3C), which represents the enzyme activity of mitochondrial dehydrogenase, also an index of cell respiration/metabolism. In addition, further MTT assay indicated that treatment of these signaling regulators alone for 1 hour at current dose was not able to efficiently affect metabolism in HUVECs, except for PD98059 which induced a slight metabolic inhibition (data not shown). Furthermore, erianin at those doses induced an activation of JNK/SAPK in HUVECs, as shown in Figure 4 with Western blotting assay for JNK/SAPK phosphorylation. Taking these data together, erianin caused a JNK/SAPK-dependent metabolic inhibition in HUVECs.

Further experimental data indicate that decreases of ATP, lactate production and glucose consumption participated in this JNK/SAPK-dependent metabolic inhibition. Erianin exposure induced dose-dependent intracellular ATP decreases, with 43.9%±7.2% of control for 12 hours of 10 nM erianin treatment and 36.1% ±6.0% for 24 hours (Figure 5A). During the first 12-hour incubation, a gradual decrease in ATP level was observed in erianin-treated HUVECs, without a sharp drop (data not shown). One-hour pretreatment with SP600125 significantly restored the erianin-induced ATP decrease, with 76.9%±6.1% of control for 12 hours of 10 nM erianin treatment and 70.3% ±8.7% for 24 hours (Figure 5A). During the first 12-hour incubation, a gradual decrease in ATP level was observed in erianin-treated HUVECs, without a sharp drop (data not shown). One-hour pretreatment with SP600125 significantly restored the erianin-induced ATP decrease, with 76.9%±6.1% of control for 12 hours of 10 nM erianin treatment and 70.3% ±8.7% for 24 hours, while among other signal regulators, only calphostin C induced a slight restoration of the reduced ATP (Figure 5A). Moreover, SP600125 pretreatment slightly but significantly suppressed the erianin-induced inhibition of lactate production and glucose consumption to a different degree: for the former, lactate production was partly restored (110.2%±3.3% for 12 hours of erianin treatment and 116.8%±6.8% for 24 hours); for the latter, a significant difference was observed only after 24-hour treatment with 93.4%±1.7% of erianin treatment (Figure 5B).

Discussion

In the present study, we investigated the inhibitory metabolic response induced by erianin, a low molecular weight natural product. Data demonstrated that erianin induced a dose-dependent endothelial metabolic inhibition, with a selectivity in endothelial cells, as shown by the acidification rate and MTT assays, which may be an important mechanism leading to its antitumor and antiangiogenic effects.
Microphysiometry Cytosensor is a non-invasive, physiological method where metabolic activity can be measured in living cells by detection of the excretion of acidic metabolic by-products, such as lactate and hydrogen ion (16). In previous publications, it was shown that cytotoxic agents used in cancer chemotherapy (17-19), and standard cytotoxic drugs with different mechanisms of action generally produced an early inhibition of the acidification rate (within 10 hours) with or without preceding transient stimulation (17). These findings are similar to the erianin-induced responses in HUVECs, indicating a metabolic cytotoxicity of erianin toward endothelial cells, which was further demonstrated by MTT assay. Moreover, the results show that endothelial glycolysis was inhibited by erianin treatment, as indicated by the assays for lactate production and glucose consumption, potentially implicating erianin in the decrease of the acidification rate.

Glycolysis is an important pathway for energy supply in endothelial cells (20), and oxidative phosphorylations occurring in mitochondrial respiration are essential to maintaining a high level of ATP (13). It is generally accepted that, to a certain extent, cellular dysfunction in response to various antitumor drugs usually result from the fact that cells fail to maintain a high level of ATP. Our data indicate that erianin could dramatically diminish the level of intracellular ATP, and oxidative phosphorylations in mitochondrial respiration is essential to maintaining a high level of ATP (13). It is generally accepted that, to a certain extent, cellular dysfunction in response to various antitumor drugs usually result from the fact that cells fail to maintain a high level of ATP. Our data indicate that erianin could dramatically diminish the level of intracellular ATP. Since the cellular level of ATP is a critical determinant for cell function and cell death (21), this fall in ATP may be an important event leading to antitumor action. In addition, given the ability of erianin to inhibit mitochondrial dehydrogenase (MTT assay), this ATP decrease may result from mitochondrial dysfunction, i.e. inhibition of oxidative phosphorylation.

The underlying mechanism in erianin-induced metabolic inhibition was also investigated in the present study by evaluating the effect of several signal regulators. The data showed that the JNK/SAPK signal was involved in erianin-induced decreases in acidification rate as well as in cell viability. Consistent with these findings, it has been recently shown that JNK/SAPK participates in the modulation of cellular metabolism directly or indirectly in various cells (22-24). Moreover, previous reports have also shown that JNK/SAPK activation plays a critical role in endothelial dysfunction induced by various antitumor or cytotoxic agents, such as colchicine, homocysteine and lipopolysaccharide (25-27). However, the precise underlying molecular mechanism, especially how the JNK/SAPK signal regulates endothelial metabolism, remains to be elucidated.

Interestingly, it should be noted that JNK/SAPK inhibition could effectively abolish the erianin-induced decrease in ATP levels. It is well known that there is a close relationship between JNK/SAPK activation and mitochondrial permeability (28,29), which plays a crucial role in cell apoptosis; but whether
JNK/SAPK directly or indirectly control mitochondrial respiration and what mechanism is involved are still unclear.

In conclusion, the present study showed that erianin induced a metabolic inhibition in HUVECs: it decreased the extracellular acidification rate, attenuated lactate production and glucose consumption, decreased cell viability and diminished intracellular ATP. Furthermore, the erianin effects on endothelial metabolism were JNK/SAPK-dependent. These data contribute to the understanding of the antitumor and antiangiogenic actions of erianin.

Acknowledgements

This work was supported by a grant from the Shanghai Key Subject Foundation, Science and Technology Development Fund (No. 00XD14022). The authors gratefully acknowledge Dr. Jin-jun Li (National Laboratory for Oncogenes and Related Genes, Shanghai Cancer Institute, P.R. China) for excellent support and helpful discussions.

References