Research Article

Arctigenin, a Potent Ingredient of Arctium lappa L., Induces Endothelial Nitric Oxide Synthase and Attenuates Subarachnoid Hemorrhage-Induced Vasospasm through PI3K/Akt Pathway in a Rat Model

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Upregulation of protein kinase B (PKB, also known as Akt) is observed within the cerebral arteries of subarachnoid hemorrhage (SAH) animals. This study is of interest to examine Arctigenin, a potent antioxidant, on endothelial nitric oxide synthase (eNOS) and Akt pathways in a SAH in vitro study. Basilar arteries (BAs) were obtained to examine phosphatidylinositol-3-kinase (PI3K), phospho-PI3K, Akt, phospho-Akt (Western blot) and morphologic examination. Endothelins (ETs) and eNOS evaluation (Western blot and immunostaining) were also determined. Arctigenin treatments significantly alleviates disrupted endothelial cells and tortured internal elastic layer observed in the SAH groups ($p < 0.01$). The reduced eNOS protein and phospho-Akt expression in the SAH groups were relieved by the treatment of Arctigenin ($p < 0.01$). This result confirmed that Arctigenin might exert dual effects in preventing SAH-induced vasospasm through upregulating eNOS expression via the PI3K/Akt signaling pathway and attenuate endothelins after SAH. Arctigenin shows therapeutic promise in the treatment of cerebral vasospasm following SAH.

1. Introduction

Delayed ischemic neurological deficit and acute cerebral ischemia subordinate to subarachnoid hemorrhage (SAH) have become a serious and fatal subcategory of stroke in patients following a ruptured aneurysm [1–4]. Connolly et al. stated the mortality rate in SAH patients ranged from 27% to 44%, and almost half of SAH patients survive with serious sequel of cognitive and functional impairment [5, 6]. Despite more than a half century of studies trying to correct and prevent vasospasm, so far there is no definitive treatment to reverse this devastating condition [7–10].

A mounting body of both direct and indirect evidence shows that spasmogens or ligands are critical in the development and maintenance of cerebral vasospasm [11–15]. Basic and cellular studies also imply two major hypotheses on cerebral vasospasm: one focuses on the synergic roles of nitric oxide (NO), nitric oxide synthase (NOS) [16–21], and endothelin-1 (ET-1) [11, 20, 22–25] and the other on intracellular signal transduction [26–30]. The putative importance of NO/ET and mitogen-activation protein kinase has not been fully emphasized; even its role in the genesis of cerebral vasospasm has been unclear. Owing to lack of effective therapies to halt this condition in SAH patients, continuous studies have focused on the pathogenesis of SAH.
Nitric oxide (NO), an effective endogenous vasodilator, is essential to vascular homeostasis and angiogenesis [31, 32]. Endothelial NO synthase is phosphorylated by various stimulations including activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway in endothelial cells [16]. Likewise, endothelin-1 (ET-1), known as a potent vasoconstrictor and promitogen, is implicated in the pathogenesis of various cardiovascular, renal, pulmonary, and central nervous system disorders [33–37]. Through coupling with ETA, a G protein receptor, ET-1, mediates vasoconstriction, whereas with ETB, it exerts a vasodilatation effect through the release of nitric oxide (NO) and prostacyclin [38]. Recent studies suggest that ET-1 stimulates the proliferation of vascular smooth muscle cells and adjudicates the interactions between leukocytes and cerebrovascular endothelium [10]. Through binding to distinct receptors, ET-1 is believed to play a critical role on mediating a variety of vascular diseases.

Arctigenin, an extract from *Arctium lappa* L., has been reported to have a variety of pharmacological activities including antioxidant, anti-inflammatory, antiproliferative, and antiviral activity [34, 39–42]. It has been shown potent in vitro anti-influenza A virus and neuroprotective against Japanese encephalitis in a mouse model [43]. In a rodent study of transient middle cerebral artery occlusion, Arctigenin was demonstrated to be neuroprotective by suppressing microglia activation and decreasing IL-1β and TNF-α expression as well as ameliorating memory impairment in Alzheimer’s disease in vitro study [44, 45].

Taking these findings together, it is reasonable to hypothesize that Arctigenin is able to reverse basilar artery spasm through activating phospho-Akt and subsequent increased eNOS expression. We used two-hemorrhage SAH model, measured the diameter of basilar artery, and examined the expression of ET-1, eNOS, and phospho-Akt in the basilar artery following SAH.

2. Methods

2.1. Materials. Arctigenin, C_{21}H_{24}O_{6}, an extract from *A. lappa* plant, was bought from Excel Biomedical Inc., Neihu Dist, Taipei 114, Taiwan, authorized by Cayman Chemical. Polyclonal anti-rat Anti-PI3K antibody (PI3K (P85) antibody), anti-phospho-PI3K antibody (phospho-PI3K (P85) (Tyr458)/(p55) (Tyr199) antibody), anti-Akt antibody (Akt antibody), and anti-phospho-Akt antibody (phospho-Akt (Ser473)) were obtained from Cell Signaling Technology (Beverly, MA 01915, USA). Horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG was purchased from R&D Systems, Inc. (Minneapolis, MN 55413, USA). CNM protein extraction kits were from Biochain (Hayward, CA 94545, USA). Arctigenin in a minipump was prepared by Ms. Wu SC (Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, ROC), and 0.9% saline was used as a vehicle.

2.2. Induction of Experimental SAH. Fifty-four male Sprague-Dawley rats, weighing between 340 and 440 g (NLAC, Education Research Resource, National Laboratory Animal Center, Taiwan), were used in this study. All the experimental protocols were authorized and superintended by the University of Kaohsiung Medicine Animal Research Committee and were compliant with the Declaration of Helsinki (1964). A modified double hemorrhage SAH model was employed [46]. The rats received anesthesia by an intraperitoneal injection of 7 mg/kg Zoletil 50 (VIRBAC, L.I.D., Carros 60516, France). 0.1 mL/100 gm fresh arterial blood was withdrawn from tail artery and injected into the cisterna magna using a stereotactic apparatus (Stoelting, Wood Dale, IL 60191, USA). After the induction, animals were placed in ventral recumbent position for 30 min. to let ventral blood clot formation. After monitoring for respiratory distress and giving mechanical ventilation if necessary, the animals were returned to the vivarium until becoming fully awake. A habitat with a 12 h light-dark cycle and access to food and water ad lib was offered. To perform the induction of 2nd SAH, the animals received repeated injection of SAH at 48 h after 1st SAH to maintain the tendency of delayed vasoconstriction. Thereafter, the animals received perfusion-fixation 72 h after 2nd SAH.

2.3. General Design of Experiments and Treatment Groups. The animals were randomly assigned into the following subgroups (9 rats/group): (1) sham operated (no SAH), (2) SAH only, (3) SAH-plus vehicle, SAH rats receiving Arctigenin treatment (4) 50 μM/kg/day, (5) 150 μM/kg/day, and (6) 450 μM/kg/day. The dosage was adjusted according to the study of a rabbit arterial ring test. The first injection was given at 1 hr after induction of SAH by using an osmotic minipump (Alzet corp, Palo Alto, CA 94301, USA) intraperitoneally. The animals were sacrificed by perfusion-fixation 72 h after SAH. Cortical tissue samples were obtained by means of placing a 20-gauge needle inserted 5 mm in depth into the skull bone through a burr hole craniectomy at a 24 h interval.

2.4. Perfusion-Fixation. By the end of the study, the animals were reanesthetized by administration of Zoletil 50 (7 mg/kg). The femoral artery was catheterized to monitor blood pressure and obtain blood to determine arterial blood gas, Na^+, K^+, GOT, and GPT levels. Perfusion-fixation was performed as opening the thorax, and the left ventricle was cannaled with a NO18 catheter by clamping the descending aorta and opening the right atrium simultaneously. 100 mL 0.01 M phosphate buffer (pH 7.4) was perfused followed by fixation with 100 mL 2% paraformaldehyde in the PBS solution at 36 °C under a pressure of 80 mm Hg. The harvested brain was immersed in a fixative at 4°C overnight. Visual inspection made sure that formed clots overlay the basilar artery (BA) in all SAH animals.

2.5. Measurement of Basilar Artery (BA) Cross-Sectional Area. Five selected cross-sections from the BA of each animal were randomly analyzed by an examiner blinded to the treatment groups. The tissues were frozen instantly and cut into 25 μm-thick sections (Reichert-Jung Ultracut E ultramicrotome). They were then stained with hematoxylin and eosin for video-assisted microscopy and the analysis of BA cross-sectional area.
2.8. Determination of ET-1. 
To measure ET-1 levels, tissue sections were stained for ET-1 using a commercial kit (R&D Systems, Minneapolis, MN 55413, USA). The tissue sections were incubated with an antibody specific for ET-1, followed by visualization with a secondary antibody conjugated to horseradish peroxidase. 

2.9. Determination of Nitric Oxide Synthase (NOS). 
NOS activity was determined using a commercial kit (Cayman Chemical, Ann Arbor, MI 48105, USA). The tissue samples were incubated with substrate L-arginine in the presence or absence of NOS inhibitor NG-nitro-L-arginine methyl ester (LNAME). The response was measured by quantifying the alteration of L-arginine to L-citrulline. 

2.6. Hemodynamic Measurements. 
Heart rate, blood pressure, and rectal temperature were monitored in the animals before and after Arctigenin administration. Heart rate was monitored with a tail-cuff method at intervals of 12 h after the induction of SAH by using a tail-cuff method (SCI1000 Single Channel System, Hatteras Instruments, NC 27518, USA) and a rectal thermometer (BIO-BRET-2-ISO, FL 33780, USA). 

2.7. Immunostaining of Basilar Artery (BA) with NOS Antibody. 
Video-assisted microscope (×400) was used to identify NOS within endothelial cells. 

2.8. Determination of ET-1. 
On the third day subsequent to induction of hemorrhage, the ET-1 levels in CSF were determined by a commercially available ELISA kit (Immuno-Biological Lab. Co., Ltd., Gunma 375-0005, Japan). The upper limit of quantification was 2000 pg/mL for ET-1 as indicated in the supplier’s instruction. 

2.9. Determination of Nitric Oxide Synthase (NOS). 
The NOS bioactivity of isolated basilar arteries was detected by quantifying the alteration of L-arginine to L-citrulline. A commercially available kit (Bioxytech NOS Assay Kit, Oxis International, CA 90210, USA) was used to examine NOS. Briefly, protein extracted from vessels was incubated with radiolabeled L-arginine in the presence or absence of 1 mmol/L NOS inhibitor NG-nitro-L-arginine methyl ester. The response was ended by adding of 50 mmol/L HEPES buffer containing 5 mmol/L EDTA. Radiolabeled L-citrulline was counted after the removal of excess L-arginine with equilibrated resin and centrifugation at 10,000 rpm. 

Table 1: Physiological parameters among the experimental groups. 

| Group          | Body weight (gm) | Systolic Blood pressure | GOT/GPT (IU/L) | BUN/Cr (mg/dL) | pH    | Temperature (°C) |
|----------------|------------------|-------------------------|----------------|----------------|-------|------------------|
| Sham           | 374 ± 24         | 104 ± 12                | 9.2 ± 3.1/7.1 ± 2.8 | 4.0 ± 2.3/0.23 ± 0.14 | 7.36 ± 0.04 | 36.5 ± 0.33     |
| SAH            | 360 ± 31         | 111 ± 8                 | 10.2 ± 4.0/11.2 ± 3.0 | 9.4 ± 1.3/0.55 ± 0.14 | 7.37 ± 0.10 | 36.1 ± 0.41     |
| SAH + Vehicle  | 359 ± 46         | 102 ± 12                | 12.4 ± 4.0/10.6 ± 3.8 | 10.2 ± 3.5/0.48 ± 0.16 | 7.38 ± 0.15 | 36.6 ± 0.42     |
| SAH + Arctigenin 50 µM/kg/day | 366 ± 49    | 108 ± 13                | 10.8 ± 4.2/13.4 ± 4.8 | 10.4 ± 4.3/0.88 ± 0.35 | 7.36 ± 0.14 | 36.8 ± 0.32     |
| SAH + Arctigenin 150 µM/kg/day | 368 ± 36     | 100 ± 12                | 12.6 ± 3.8/7.8 ± 4.2 | 11.6 ± 4.1/1.3 ± 0.38 | 7.36 ± 0.41 | 36.6 ± 0.51     |
| SAH + Arctigenin 450 µM/kg/day | 370 ± 42     | 94 ± 34                 | 13.5 ± 2.6/7.8 ± 3.8 | 12.4 ± 2.6/0.86 ± 0.43 | 7.38 ± 0.13 | 36.7 ± 0.13     |

Results are expressed as the mean ± SEM, n = 9; p < 0.01 versus SAH condition by one-way analysis of variance (ANOVA) analyses followed by Mann-Whitney U-test.

2.11. Statistical Analysis. 
Data are expressed as the means ± standard deviation. For group comparisons, all statistical analyses were determined with one-way analysis of variance, with the Bonferroni post hoc test and Mann-Whitney U test. Difference, at a probability value less than 0.01, was considered significant.

3. Results
3.1. General Observation. 
Throughout this study, all animals showed no significant differences in the physiological parameters recorded, including glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), blood urea nitrogen (BUN), creatinine, pH, and arterial blood gas analysis among all groups (Table 1). Slight lower systemic blood pressure was observed in the 450 µM/Kg/day Arctigenin treatment SAH group. It proved that continued
Arctigenin pumping in the selected dosage has a number of pleiotropic effects but is devoid of hepatic and renal toxicity.

3.2. Morphometric Analysis. The lumen area was significantly increased while the radial wall thickness, which has been considered an index of chronic inflammation, was decreased in both of the Arctigenin groups when compared with those of the SAH groups. Lumen cross-sectional areas in the sham-operated, SAH-only vehicle, and 50/150/450 μM/kg Arctigenin treatment SAH groups were 0.28±0.014, 0.14±0.018, 0.15±0.023, 0.17±0.022, 0.24±0.032, and 0.25±0.025 (mean ± SD) mm², respectively (Figure 1, bottom panel).

3.3. ELISA for ET-1. The concentration of ET-1 in the CSF reached the peak at 48 hr after the induction of SAH; 408±35 pg/mL was found as the baseline value in the sham-operated animals. The levels of ET-1 were significantly decreased by 22%, 51%, and 46% in 50 μM/kg/day, 150 μM/kg/day, and 450 μM/kg/day Arctigenin administration respectively, as compared with 2414±85 pg/mL of the SAH + vehicle group (Figure 2).

3.4. Activated eNOS. Radiolabeled L-citrulline analysis showed higher eNOS protein in the healthy controls. Treatment with 50/150 μM/Kg Arctigenin did cause a significant increase in the bioactivity of the eNOS protein compared with rats of the SAH groups (Figure 3). Treatment of 450 μM Arctigenin increased the eNOS expression 1.4-fold when compared with SAH + vehicle rats, which corresponds to the observation in the BA eNOS immunostaining (Figure 3, upper panel).

3.5. Levels of PI3k, Phosphor-PI3K (P85), Akt, and Phosphor-Akt. Even with trends for downregulation of PI3K/Akt signaling pathway following induction of SAH, there were no significant differences among the five experimental groups (Figure 4). Levels of phospho-PI3K and phospho-Akt were increased significantly in animals subjected to SAH (SAH-only, and SAH-plus vehicle treatment groups) when compared to that in healthy controls (p < 0.01, and p < 0.01 resp.; Figure 4). Treatment with Arctigenin had less effect on the levels of PI3K/Akt after SAH, while treatment with Arctigenin significantly induced the levels of the phosphor-PI3K/Akt after SAH (p < 0.01 and p < 0.01 resp., Figure 5) when compared with that of SAH-plus vehicle group.

4. Discussion
In this study, we have shown that Arctigenin, a potent antioxidant, is able to increase Akt activation and attenuate vascular constriction in rats subjected to SAH. It is plausible that antivasospasm effects of Arctigenin account partly for simultaneous modulation of eNOS accompanying with muscarinic and β-adrenergic receptors activation. ET-1 is proven to be among the most potent causative factors of cerebral vasospasm after SAH. The biosynthesis of ET-1 is composed of conversing the natively precursor big ET-1 to...
the mature peptide ET-1 to -5 by a nonpeptide proteinase, endothelin converting enzyme (ECE) [14, 23]. Extracellular ET-1, produced in endothelial cells and glia cells, can adjust cerebrovascular tone via interactions with specific ET-A and -B receptors on the membranes of smooth muscle and endothelial cells [7, 31]. Owing to the crucial role of active ET-1, it is conceivable that inhibition of activated ETs could be amenable to the treatment of SAH-induced vasospasm. This study offers first evidence that Arctigenin exerts antivasospastic effect by means of inhibiting the production of ET-1.

NO, a potent endothelium-derived relaxant, has been well characterized in SAH-induced vasospasm. Three subtypes are included in the NOS family: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). eNOS mainly exists in the endothelium. The iNOS phenotype is detected only at low levels in humans [20]. NO plays a decisive role in modulating vascular tension and is also an important pathophysiological mediator in cardiovascular disorders and activates soluble guanylyl cyclase, which leads to the production of cGMP [39]. In Neuschmelting et al’s study, NO donors effectively reduced the production of ET-1 from porcine-cultured aortic endothelial cells [20]. In this study, expression of eNOS is induced by Arctigenin in animals subject to SAH, which may contribute to its vascular dilatation effects. In light of previous findings showing that NO modulates the production of ET-1, the result of the study disclosing Arctigenin exerts the vasodilation effect through both the suppression of ET-1 and enhancement of NOS merits further evaluation.

Dimmeler et al. demonstrated that Akt phosphorylated the Serine 1177 site of eNOS protein and enhanced eNOS activity. Through inhibiting the PI3K/Akt pathway, it led to the prevention of eNOS activation [16]. Akt plays a crucial role in eNOS activity and is important in endothelial cell migration and angiogenesis, protection in ventilator-associated pulmonary injury [36]. It also protected intestinal tissue in the situation of intestinal ischemia [37]. The onset of Akt cascade is activated by tyrosine kinases, integrins, B and T cell receptors, cytokine receptors, G-protein coupled receptors, and other stimuli that promote the production of phosphatidylinositol 3,4,5 triphosphates (PIP3) by phosphoinositide 3-kinase (PI3K). There are three related isoforms of Akt: Akt1, Akt2, and Akt3. Akt1 is a key signaling protein in the cellular survival pathway. Akt2 serves as a critical signaling molecule in the insulin signaling pathway, but the role of Akt3 remains unclear [47]. Akt is a major mediator of cell survival by direct inhibition of proapoptotic signals such as the proapoptotic regulator Bad and the Fox01 and Myc family of transcription factors [16, 33]. The trafficking of T lymphocyte to lymphoid tissues is controlled by the expression of adhesion molecules related downstream of Akt [32]. In Endo et al’s study, Akt has been shown to downregulate superoxide dismutase-associated neuron apoptosis in SAH [17].

Figure 2: The effect of Arctigenin on the change in ET-1 in CSF after the induction of SAH (ELISA). Arctigenin dose-dependently attenuates ET-1 in the SAH rats, when compared with the SAH group. Data in the figure are presented as mean ± SD (n = 9). *p < 0.01, compared between the Sham-operated and the SAH group; **p < 0.01, and #p > 0.01 when compared with the SAH + vehicle group.
Ishrat et al. also reported progesterone exerts neuroprotective effect through the Akt signaling pathway [15]. In this study, expression of phospho-PI3K and phospho-Akt was dose-dependently induced by Arctigenin in animals subject to SAH, which may contribute to its vascular dilatation effects and correspond to its effect on the eNOS.

Arctigenin, as an active ingredient extracted from *A. lappa* (L.), has been used to exert many pharmacological functions such as antidiabetes, antitumor, antioxidant, and neuroprotective effect [39, 40, 44]. In this study, Arctigenin was found able to inhibit ET-1 production and enhance eNOS by the activation of the PI3k/Akt signaling pathways.

Currently, treatments for cerebral aneurysms and associated vasospasm are complicated and disappointing. Besides, patients with vasospasm have more inpatient cost and longer hospital stay, and definite effective medical treatment against vasospasm is still lacking [5]. Clinically, oral nimodipine treatment for SAH patients might improve functional outcome but not contribute to the improvement of vasospasm [6]. A phase 3 randomized trial of MASH-2 published results that magnesium sulfate did not improve clinical outcome in 1,203 patients with aneurysmal SAH [13]. Herein, we found that Arctigenin effectively inhibited PI3k/Akt pathways and increased eNOS activation. This result thus highly strengthened the potential of this natural product in its antivasospasm in the SAH study.

In summary, the results of this study show that continued administration of Arctigenin, at a selected therapeutic dosage, is safe and efficacious in the prevention of vasospasm in this experimental model and is meritorious of further investigation. Decreased levels of ET-1 as well as activated eNOS, phospho-PI3k and Akt may contribute to the anti-apoptotic and antivasoconstrictive effect of this compound. Besides, 150 μM/kg of Arctigenin is similarly effective as that of 450 μM/kg and exerts a dual effect on the Akt-related NOS pathway and ET-1 related inflammatory cascade.

5. Conclusions

The outcome of SAH patients is persistently devastating, and has changed little after decades of research and treatment focusing on cerebral vasospasm. These accumulated results arouse interest to consider the pathogenesis of SAH-induced
EBI and its effects dictating the patient’s outcome. The ultrashort activated ET-1 and NOS following SAH lead to transient vasoconstriction and a cascade of followed neuroinflammatory reactions maintains the intensity of vasoconstriction. The spasms accompanying SAH may be another complicated pathway underlying the development and maintenance of delayed vasospasm. This study shows that administration of Arctigenin, a natural ingredient, induces eNOS, and diminishes SAH-induced ET-1 related vasospasm in a rodent model of SAH. Our result also suggests that Arctigenin could prove clinically useful in treating SAH-associated morbidity.

**Abbreviations**

| Acronym  | Term                        |
|----------|-----------------------------|
| BA       | Basilar artery              |
| Caspases | Cysteine requiring aspartate proteases |
| CSF      | Cerebrospinal fluid         |
| ET-1     | Endothelin-1                |
| ETs      | Endothelins                 |
| eNOS     | Endothelial nitric oxide synthase |
| PI3K     | Phosphatidylinositol 3-kinase |
| PKB, Akt | Protein kinase B            |
| SAH      | Subarachnoid hemorrhage     |
| TNF-α    | Tumor necrotic factor-α     |
| HRP      | Horseradish peroxidase      |
| IEL      | Internal elastic lamina     |
| IL-1 & -6| Interleukins 1 and 6        |
| PBS      | Phosphate-buffered saline   |
| PGs      | Prostaglandins              |

**Conflict of Interests**

There is no conflict of interests related to this paper.

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The effectiveness of Arctigenin on the immunohistochemical study of Akt/phospho-Akt expression (western blot). Upper panel: the groups are as depicted in Figure 3. The assay showed Arctigenin induced Akt/phospho-Akt immunoreactivity in the SAH rats when compared with the SAH groups. All values are mean ± SD. * p < 0.01, comparison with the SAH group; ** p < 0.01; and # p > 0.01, compared with the SAH + vehicle group.

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