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Esculetin inhibits Ras-mediated cell proliferation and attenuates vascular restenosis following angioplasty in rats

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Abstract

The proliferation of vascular smooth muscle cells (VSMCs) induced by injury to the intima of arteries is an important etiologic factor in vascular proliferative disorders such as atherosclerosis and restenosis. Esculetin, derived from the Chinese herb *Artemisia scoparia*, is well known as a lipoxygenase inhibitor. We have investigated the inhibitory effects of esculetin on VSMC proliferation and intimal hyperplasia by balloon angioplasty in the rat. We determined, using [^{3}H]thymidine incorporation and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, that esculetin inhibited the proliferation of VSMCs via a lipoxygenase-independent pathway. Three predominant signaling pathways were identified to be inhibited by esculetin: (a) the activation of p42/44 mitogen-activated protein kinase (MAPK) and the downstream effectors of c-*fos* and c-*jun* immediate early genes by means of western and reverse transcription–polymerase chain reaction (RT–PCR) analyses; (b) the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), using the electrophoretic mobility shift assay; and (c) the activation of phosphoinositide 3-kinase (PI 3-kinase) and cell cycle progression, by western blot analysis and flow cytometric detection. Furthermore, esculetin also profoundly inhibited Ras activation, a shared upstream event of the above signaling cascades. In vascular injury studies, intraperitoneal administration of esculetin significantly suppressed intimal hyperplasia induced by balloon angioplasty. We conclude that esculetin blocks cell proliferation via the inhibition of an upstream effector of Ras and downstream events including p42/44 MAPK activation, PI 3-kinase activation, immediate early gene expression, as well as NF-κB and AP-1 activation. It also inhibits intimal hyperplasia after balloon vascular injury in the rat, indicating the therapeutic potential for treating restenosis after arterial injury.

Keywords: Esculetin; Proliferation; Smooth muscle cells; Ras; Restenosis

1. Introduction

The structure of the arterial wall and its vasoactive properties are derived mainly from VSMCs. VSMCs are involved in several pathological conditions such as neointima formation, restenosis after acute vascular injury, and chronic pathological processes such as atherosclerosis [1]. Cell proliferation is initiated by the transduction of mitogenic signals from cell surface receptors to the nucleus via an activation cascade, such as receptor protein tyrosine kinase, Ras, Raf, and p42/44 MAPK [2,3]. p42/44 MAPK is a serine/threonine kinase, which is activated by the phosphorylation of both threonine and tyrosine residues [4]. Phosphorylated p42/44 MAPK translocates into the nucleus, where it phosphorylates several nuclear transcription factors, including AP-1 and NF-κB, ultimately leading to the initiation of gene transcription, the transition of cells from a quiescent to a proliferative state, DNA synthesis, and cell division [5–7].

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Abbreviations: VSMCs, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; AP-1, activator protein-1; PI 3-kinase, phosphoinositide 3-kinase; Cdks, cyclin-dependent kinases; IκB, inhibitor κB; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EMSA, electrophoretic mobility shift assay; ECL, enhanced chemiluminescence; GST, glutathione *S*-transferase; RBD, Ras-binding domain; RT–PCR, reverse transcription–polymerase chain reaction; ROS, reactive oxygen species.

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Efforts to inhibit the proliferation of VSMCs in vascular injury models, either by modulating cellular mediators of the proliferative response or by interfering with the cell cycle machinery, have provided insights into neointima formation. Cell cycle progression is a tightly controlled event regulated by Cdks and their cyclin-regulatory subunits [8]. This activation triggers DNA synthesis and initiates cell cycle transition via phosphorylation of nuclear transcription factors of immediate early genes, such as c-*fos*, c-*jun*, and c-*myc* [9]. The AP-1 complex consists of Fos (c-fos, Fos B, Fra-1, and Fra-2) and Jun (c-jun, Jun B, and Jun D) protein homodimers or heterodimers and binds to regulatory sequences in the promoter of various target genes involved in cell growth, differentiation, and transformation [9]. Among the *fos/jun* family genes, c-*fos* and c-*jun* are the two major components of the AP-1 complex.

Recently, NF-κB has been reported to play a pivotal role in regulating gene expression controlling inflammation, cell differentiation, apoptosis, and proliferation [10]. Furthermore, the expression of NF-κB is enhanced in vascular tissue during VSMC proliferation after arterial injury [11]. Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of p50, p65, and IκB subunits. Upon activation, degradation of IκB exposes nuclear localization signals on the p50/p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene regulation. IκB degrades immediately after injury to vascular walls. These findings suggest that IκB plays a key role in regulating the activation of NF-κB in VSMC proliferation in vascular cell walls [12].

Esculetin, a coumarin derivative from *Artemisia scoparia*, is a potent non-competitive inhibitor of lipoxygenase [13]. In the present study, we examined the effect of esculetin on VSMC proliferation in response to serum. We determined the cellular signals to esculetin action such as the regulation of transcription factors AP-1 and NF-κB, the expression of immediate early genes, and cell cycle machinery through the p42/44 MAPK or PI 3-kinase-dependent signaling pathway. Furthermore, we determined the effect of esculetin on intimal thickening in a rat common carotid artery model of vascular restenosis. After these determinations, the antiproliferative mechanism of esculetin could be defined and the therapeutic potential for treating restenosis after arterial injury evaluated, in detail.

2. Materials and methods

2.1. Cell culture

VSMCs were isolated from the thoracic aorta of Wistar rats and cultured in DMEM supplemented with 10% FBS. Passages before the 7th generation were used for the experiments.

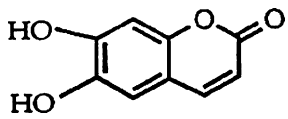

Fig. 1. Structure of esculetin.

2.2. [^{3}H]Thymidine incorporation assay

Confluent VSMCs were trypsinized and seeded at 1.0×10^4 cells/well into 96-well plates. Cells were preincubated with or without esculetin (Fig. 1) (Aldrich Chemical Co.; 98% purity), treated with 10% FBS for 48 hr, and then harvested for the detection of DNA synthesis. Before the harvest, cells were incubated with [^{3}H]thymidine (1 μCi/mL, Amersham Pharmacia) for 16 hr and then were processed and harvested with a Filter-Mate (Packard); the incorporated radioactivity was then determined.

2.3. MTT assay method

The alteration of cell number was determined by the colorimetric MTT assay. In brief, after the cells were treated with or without the indicated agent and/or serum for 48 hr, they were incubated with 0.5 mg/mL of MTT (Sigma) for 1 hr and lysed with dimethyl sulfoxide for the determination of absorbance density, using an ELISA reader.

2.4. Western blot analysis

Cultured VSMCs were lysed in a standard lysis buffer. The cell lysate was centrifuged at 2000 *g* for 10 min at 4°, and the supernatant was used for western blot analysis as described previously [14]. Membranes were incubated overnight at 4° with the primary antibodies p42/44 MAPK, phosphorylated p42/44 MAPK, IκB-α, phosphorylated IκB-α, and cyclin D1 (Santa Cruz Biotechnology), and phosphorylated Akt (Upstate Biotechnology), diluted 1:1000 in PBS. After several washing procedures and conjugation with secondary antibodies, the membranes were subjected to ECL (Amersham) to measure protein expression.

2.5. Detection of AP-1 and NF-κB activity

The DNA-binding activities of AP-1 and NF-κB were determined using EMSA. VSMCs were activated with stimulants for either 4 hr (AP-1) or 1 hr (NF-κB), and then were collected with a cell scraper. Nuclear extracts were prepared and applied to the gel shift assay as described previously [15]. Briefly, nuclear extracts (2 μg) were incubated with a 35-bp double-stranded ^{32}P-labeled probe encoding the AP-1 consensus sequence (5′-CGC TTG ATG AGT CAG CCG GAA-3′) or the κB consensus sequence (5′-AGT TGA GGG GAT CCC CCC AGG C-3′) in binding buffer containing 10 mmol/L of Tris–HCl, 40 mmol/L of NaCl, 10% glycerol, 1 mmol/L of EDTA,

1 mmol/L of dithiothreitol, 1% Nonidet P-40, 1% deoxycholate, and 3 μg/mL of polydeoxyinosinic-deoxycytidylic acid at room temperature for 30 min. Then samples were applied to native 5% polyacrylamide gels and analyzed by autoradiography. For the competition assay, a 20-fold molar excess of unlabeled consensus oligonucleotide was added 30 min prior to the addition of the labeled probe. Components of NF-κB proteins were identified by a supershift assay using antibodies against p50 or p65; AP-1 proteins were identified by using antibodies against c-jun or c-fos.

2.6. RT–PCR analysis of c-fos and c-jun mRNA expression

Total RNA isolation and RT–PCR were performed as described previously [16]. The sequences of the primers for amplifying c-fos, c-jun, and GAPDH cDNA are as follows: c-fos sense, 5′-GTAGAGCAGCTATCTCCTGA-3′; c-fos antisense, 5′-TCCACATCTGGCACAGAGC-3′; c-jun sense, 5′-AACGACCTTCTACGACGATG-3′; c-jun antisense, 5′-GCAGCGTATTCTGGCTATGC-3′; GAPDH sense, 5′-ACCACAGTCCATGCCATCAC-3′; and GAPDH antisense, 5′-TCCACCACCCTGTTGCTGTA-3′.

2.7. Flow cytometric analysis

Cells were synchronized at the G_0 phase by serum depletion for 48 hr. Then the cells were incubated in the absence or presence of the indicated agent, after which 10% FBS was added to allow the progression of the cell cycle. After a 24-hr treatment, cells were trypsinized, fixed, and stained with propidium iodide. The cell cycle distribution was determined using FACScan and analyzed by CellQuest software.

2.8. Determination of Ras activity

The active GTP-bound form of Ras was detected by using a GST fusion protein corresponding to the RBD of Raf-1 bound to glutathione-agarose according to the protocol of the manufacturer (Upstate Biotechnology). The proteins were resolved by 12% SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Ras was detected by immunoblot analysis with anti-Ras antibody.

2.9. Restenosis model and histological examination

Wistar rats were anesthetized with sodium chlorohydrate (37 mg/kg) administered intraperitoneally, and a 2F Fogarty arterial embolectomy balloon catheter was inserted into the left carotid artery. Esculetin (10 mg/kg/day) was injected intraperitoneally into the rat 3 days before to 14 days after the vascular injury. On day 14 after the injury, the rats were killed by intraperitoneal administration of pentobarbital (60 mg/kg), and the left and right (control group) carotid arteries were removed, fixed with 4% formaldehyde, and stained with hematoxylin-eosin. Six sections on each slide were analyzed by computerized morphometry (NIH image) by an investigator blinded to the type of the experimental group. The degree of neointimal thickening was expressed as the ratio of the neointima area to the area of the media (N/M).

2.10. Statistical analysis

Data are presented as the means ± SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way ANOVA followed by a *t*-test; *P* values less than 0.05 were considered significant.

3. Results

To examine the effect of esculetin on the regulation of cell growth, [^{3}H]thymidine incorporation and MTT assay methods were used to determine DNA synthesis and cell number, respectively. As demonstrated in Fig. 2, esculetin inhibited the serum-induced increase of DNA synthesis and cell number in a concentration-dependent manner,

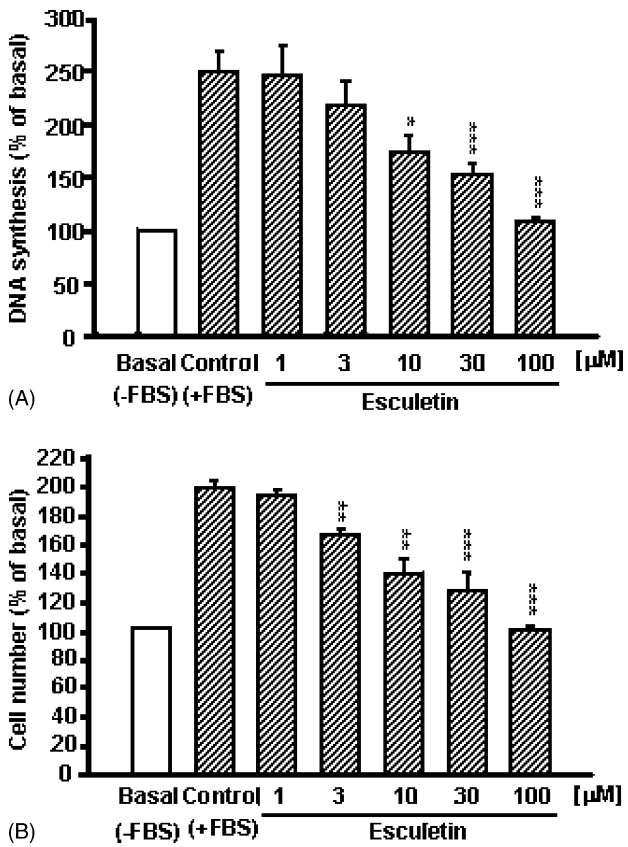

Fig. 2. Effect of esculetin on the regulation of proliferation. Cells were treated with or without esculetin, and then vehicle or FBS (10%, v/v) was added for 48 hr. After the incubation period, DNA synthesis and cell number were assessed using [^{3}H]thymidine incorporation (A) and the MTT assay (B), respectively. Data are expressed as means ± SEM of six determinations. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$, compared with the control.

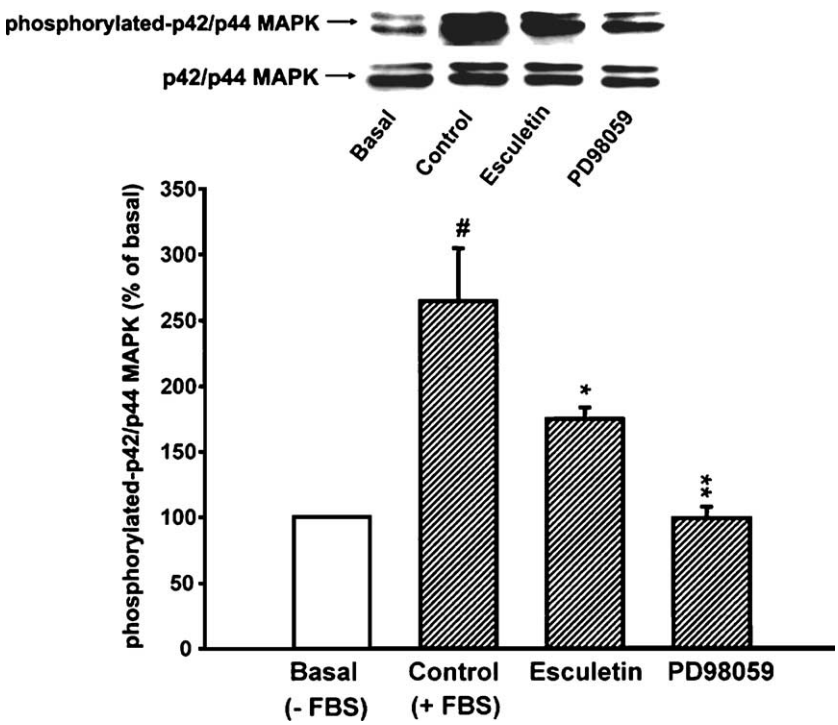

Fig. 3. Effect of esculetin and PD98059 on p42/44 MAPK phosphorylation. Cells were incubated in the absence or presence of esculetin (100 μM) or PD98059 (30 μM) for 1 hr, and vehicle or FBS was added to the cells for another 10 min. Cells were harvested for the detection of phosphorylated-p42/44 MAPK and total p42/44 MAPK using western blotting. Data are expressed as means ± SEM of four determinations. Key: (#) $P < 0.05$, compared with basal, and (*) $P < 0.05$ and (**) $P < 0.01$, compared with control.

suggesting an antiproliferative action of esculetin. Furthermore, esculetin showed little influence on the release reaction of lactate dehydrogenase (data not shown), revealing that its antiproliferative action was not due to a cytotoxic effect.

We measured the effect of esculetin on serum-induced activation of p42/44 MAPKs, which act downstream of several types of mitogenic stimuli and upstream of several transcriptional events [4]. The phosphorylated p42/44 MAPKs were examined to determine MAPK activation. The data showed that serum induced a profound increase of p42/44 MAPK activation. Both esculetin and 2′-amino-3′-methoxyflavone (PD98059), a selective MAPK kinase (MEK) inhibitor, significantly inhibited the effects of serum addition (Fig. 3), revealing that esculetin behaves as an upstream regulator of p42/44 MAPK activation.

The binding of AP-1, the dimeric transcription factor composed of Fos and Jun subunits, to a common DNA site (AP-1-binding site) also stimulates VSMC proliferation. In the present work, esculetin and PD98059 reduced the serum-mediated induction of c-*fos* and c-*jun* gene expression (Fig. 4A). We also determined the effect of esculetin on AP-1 binding activity. The results demonstrated that serum significantly increased AP-1 binding activity (Fig. 4B, lane 2); both esculetin and PD98059 significantly inhibited the serum-induced effect (Fig. 4B, lanes 3 and 4, respectively). Furthermore, the binding specificity was confirmed by competition with the unlabeled AP-1 probe added in molar excess (Fig. 4B, lane 5). The supershift assays suggested the existence of an additional AP-1-related complex against c-fos and c-jun (Fig. 4B, lanes 6 and 7, respectively).

It has been well established that NF-κB activity is regulated by IκB proteins, and that the phosphorylation and degradation of IκB-α result in the activation of NF-κB. As shown in Fig. 5, the exposure of quiescent cells to serum for 10 and 60 min stimulated a profound increase of IκB-α phosphorylation and subsequent degradation, respectively; both esculetin and PD98059 significantly inhibited the serum-induced effects (Fig. 5A and B). Furthermore, both esculetin and PD98059 also effectively inhibited the serum-induced DNA-binding activity of NF-κB (Fig. 5C).

By means of flow cytometric assessment we found that about 96% of the cells arrested in the G_0/G_1 phase of the cell cycle when the cells were made quiescent by the deprivation of serum. After 10% FBS supplementation for 24 hr, a significant increase of cells entering into the S and G_2/M phases (21%) was observed. However, in the presence of esculetin, the serum-induced effect was inhibited profoundly (8% in the S and G_2/M phases). We also examined cyclin D1 expression. The data showed that serum induced cyclin D1 expression; however, esculetin completely abolished this action of serum (Fig. 6). These results suggest that esculetin could effectively inhibit serum-induced cell cycle progression so that the cells remained in a quiescent state. Interestingly, in the present study, PD98059 and U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; an MEK inhibitor] had

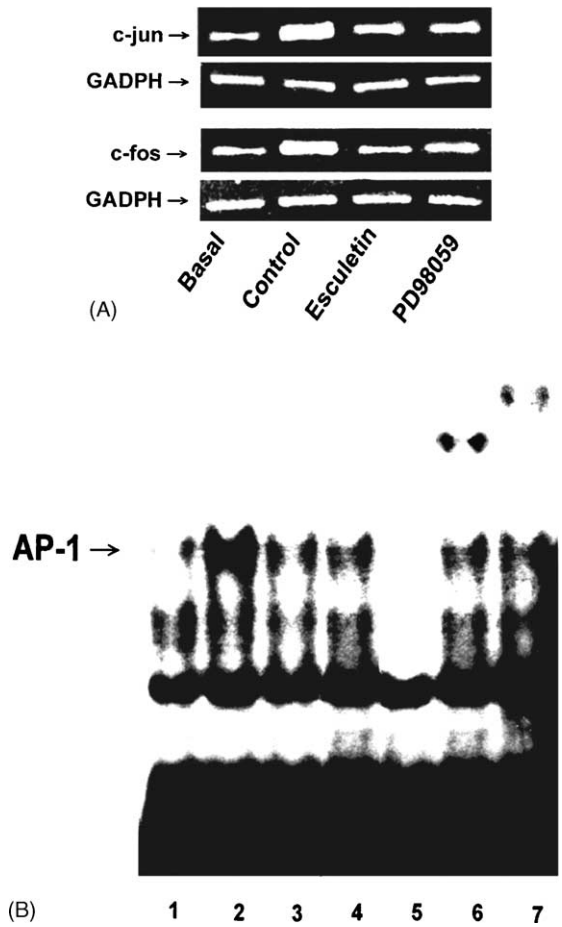

Fig. 4. Effect of esculetin and PD98059 on c-fos and c-jun mRNA expression and AP-1 DNA binding activity. Cells were incubated in the absence or presence of esculetin (100 μM) or PD98059 (30 μM) for 1 hr, and vehicle or FBS was added to the cells for another 1 (A) or 4 (B) hr. After the treatment, cells were harvested and nuclei were extracted for the detection of c-fos and c-jun mRNA levels (A) and AP-1 DNA binding activity (B) (lane 1, basal; lane 2, control; lane 3, esculetin; lane 4, PD98059; lane 5, competitor; lane 6, c-fos antibody; and lane 7, c-jun antibody) using RT–PCR and electrophoretic mobility shift assay, respectively.

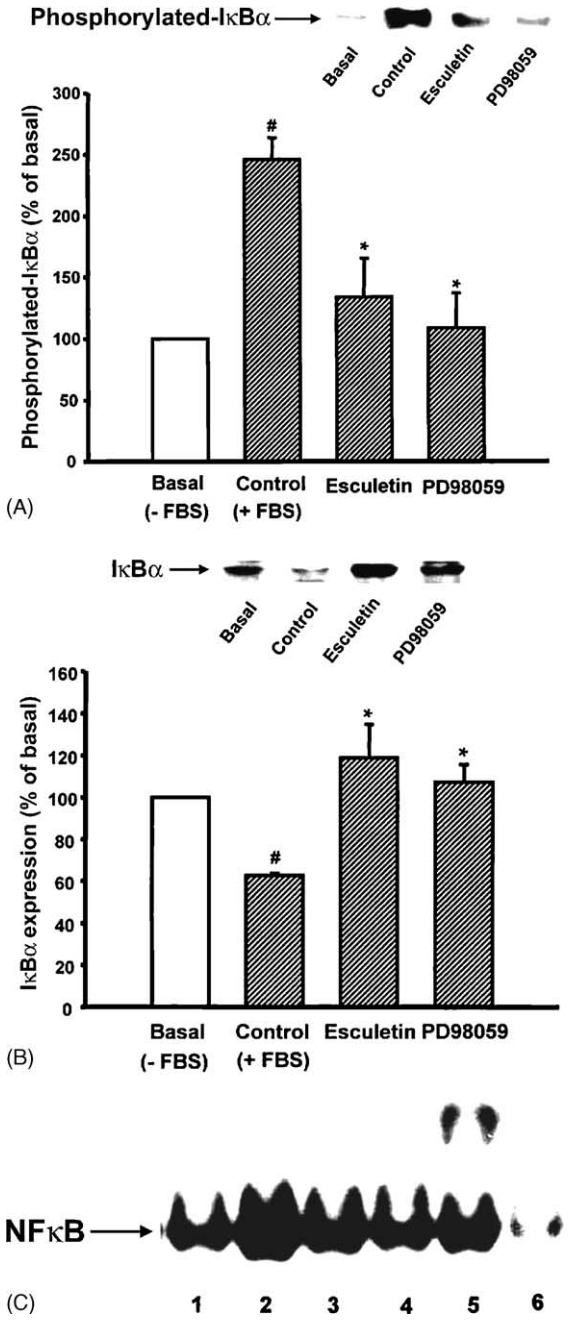

Fig. 5. Effect of esculetin and PD98059 on the phosphorylation and degradation of IκB-α and NF-κB DNA binding activity. Cells were treated with or without the indicated agent (100 μM esculetin, 30 μM PD98059) for 1 hr, and then vehicle or FBS was added to the cells for another 10 min (A) or 1 hr (B and C). After the treatment, cells were harvested for the detection of IκB-α phosphorylation and degradation, or the nuclei were extracted for the detection of NF-κB nuclear translocation (lane 1, basal; lane 2, control; lane 3, esculetin; lane 4, PD98059; lane 5, p65 antibody; and lane 6, competitor). Data are expressed as means ± SEM of three determinations. Key: (#) $P < 0.05$ and (*) $P < 0.05$, compared with basal and control, respectively.

little influence on the serum-induced increase of cyclin D1 expression (Fig. 6).

PI 3-kinase is a member of a ubiquitously expressed enzyme family. To determine the effect of esculetin and PD98059 on PI 3-kinase activity, we measured their actions on Akt/PKB phosphorylation, which is downstream of PI 3-kinase, after the growth stimuli. As demonstrated in Fig. 7, serum induced a marked increase of Akt/PKB phosphorylation that was inhibited completely by esculetin and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; a selective PI 3-kinase inhibitor]. However, PD98059 significantly increased rather than decreased the amount of phosphorylated Akt/PKB (Fig. 7). These data reveal that esculetin and LY294002 inhibit, while PD98059 stimulates, serum-induced PI 3-kinase activation.

There are several lines of evidence suggesting that activated Ras stimulates a multitude of downstream signaling cascades [17]. Because of the broad spectrum of esculetin on the inhibition of serum-induced effects, we examined the activity and total protein expression of Ras in the present work. As shown in Fig. 8, serum caused a rapid activation of Ras; however, esculetin and α-hydroxyfarne-

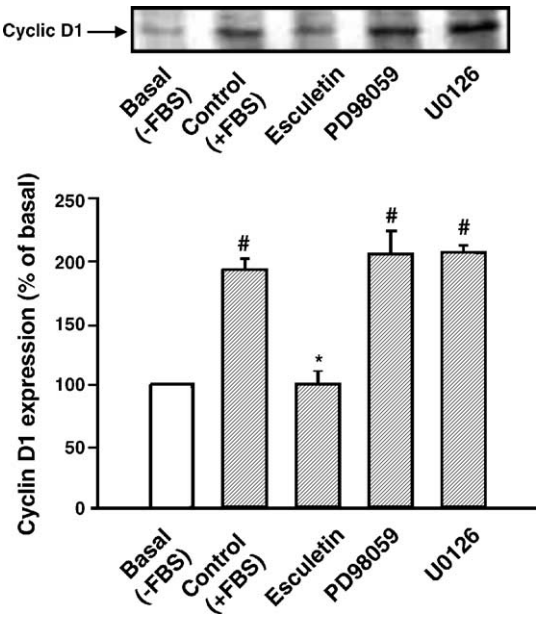

Fig. 6. Regulation of cell cyclin D1 expression. Cells were treated with or without esculetin (100 μM), PD98059 (30 μM), or U0126 (1 μM) for 1 hr, and then vehicle or FBS was added to the cells for another 24 hr. After the above treatment, cells were harvested for the detection of cyclin D1 expression using western blot analysis. Data are expressed as means ± SEM of three determinations. Key: (#) $P < 0.05$ and (*) $P < 0.001$, compared with basal and control, respectively.

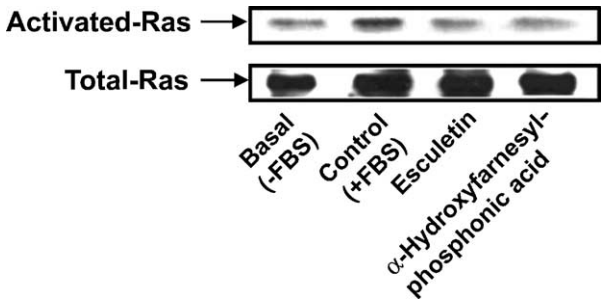

Fig. 8. Effect of esculetin on pan-Ras activity. Cells were incubated in the absence or presence of the indicated agent for 1 hr, and vehicle or FBS was added to the cells for another 5 min. Cells were harvested, and pan-Ras activity was measured. N = 3.

sylphosphonic acid, a Ras farnesyltransferase inhibitor, profoundly inhibited this serum-induced effect (Fig. 8).

Wistar rats were injected intraperitoneally with vehicle or esculetin (10 mg/kg/day) beginning 3 days before balloon angioplasty. On day 14 after angioplasty, the animals were killed and examined for evidence of vascular injury and intimal thickening. As demonstrated in Fig. 9, the carotid artery without balloon angioplasty exhibited little change in any area or in thickness (Fig. 9A), while the injured vessel showed a profound neointimal thickening (Fig. 9B). However, there was a profound reduction in neointimal thickness in the esculetin-treated animals (Fig. 9C and D).

4. Discussion

Herbal medicines used to treat Chinese people for thousands of years are now being manufactured in many countries as drugs with standardized quality and quantity of ingredients. In our laboratory, we have examined many

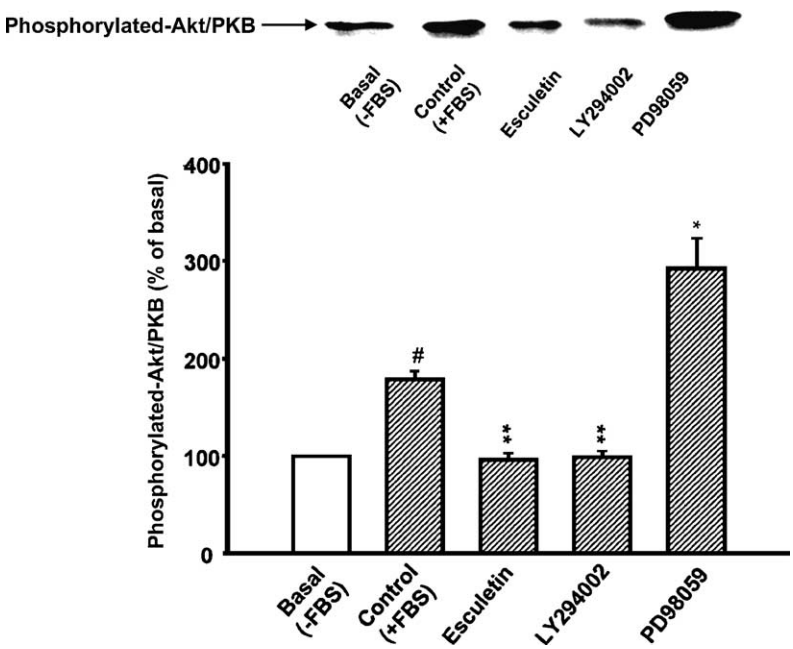

Fig. 7. Effect of esculetin on Akt/PKB phosphorylation. Cells were treated with or without esculetin (100 μM), LY294002 (10 μM), or PD98059 (30 μM) for 1 hr, and then vehicle or FBS was added to the cells for another 15 min. After the above treatment, cells were harvested for the detection of phosphorylated-Akt/PKB using western blot analysis. Data are expressed as means ± SEM of five determinations. Key: (#) $P < 0.05$, compared with basal, and (*) $P < 0.01$ and (**) $P < 0.001$, compared with control.

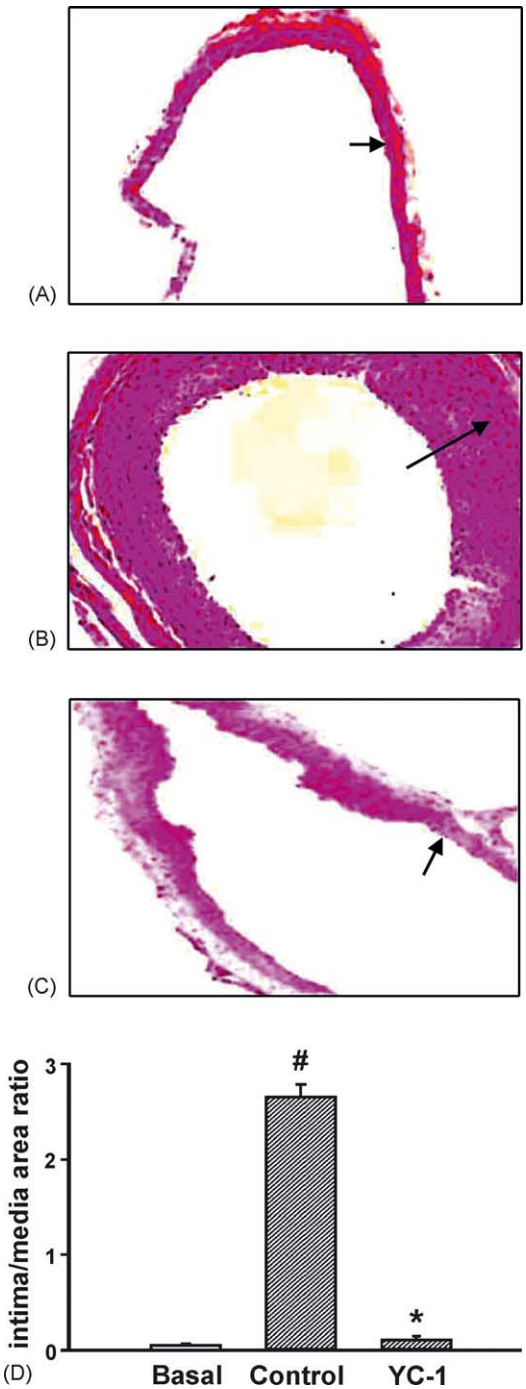

Fig. 9. Responses of rat carotid arteries to balloon injury. Representative observations are sections from an uninjured vessel (A), a balloon-injured vessel (B), and an esculetin-treated balloon-injured vessel (C). Data are also quantified by the neointima/media ratio of common carotid arteries after balloon injury from each group of animal studies (D). Arrows indicate the elastic laminae over which are the neointimal layers. All pictures were taken at 200× magnification. Data are expressed as means ± SEM of six animals in each group. Key: (#) $P < 0.05$ and (*) $P < 0.01$, compared with basal and control, respectively.

purified compounds and crude extracts from herbal medicines of Chinese medicinal prescriptions to determine their active components against VSMC proliferation, a causative factor in several vascular diseases. In our present work, we found that esculetin exhibits a profound antiproliferative effect in VSMCs. We, therefore, established a cascade-based study model to explore the antiproliferative mechanism.

Esculetin is famous as a potent non-competitive inhibitor of lipoxygenase [13]. In this study, using [^{3}H]thymidine incorporation, MTT assay, and flow cytometric assessment, esculetin was found to exhibit a marked antiproliferative effect in VSMCs. However, we doubt that the antiproliferative effects of esculetin are mediated by the inhibition of lipoxygenase activity for two reasons. First, the IC_{50} value needed for the antiproliferative effect (about 10 μM) is more than 30 times that needed for lipoxygenase inhibition (about 0.3 μmol/L). Second, the lipoxygenase metabolites 12- and 15-HETE could neither induce the proliferative effect [118.7 ± 3.5 and $102.3 \pm 2.4\%$, respectively, compared with the control ($100 \pm 0\%$), $N = 4$, $P > 0.05$] nor reverse the antiproliferative action of esculetin (47.3 ± 3.8 and $49.2 \pm 3.1\%$ inhibition, respectively, compared with that of esculetin alone which inhibited the action of serum by $51.4 \pm 4.0\%$, $N = 4$, $P > 0.05$), suggesting that the antiproliferative effect of esculetin was not via the inhibition of cellular lipoxygenase.

It has been suggested that several second messengers such as cyclic AMP, cyclic GMP, and some prostaglandins could inhibit the proliferation of VSMCs [18–20]. In the present study, esculetin could not effectively induce the formation of cyclic AMP and cyclic GMP ($105 \pm 11\%$ compared with the control value of $100 \pm 0\%$ cyclic AMP formation, $N = 3$; $102 \pm 14\%$ compared with the control value of $100 \pm 0\%$ cyclic GMP formation, $N = 3$), nor could exposure to indomethacin, a potent cyclooxygenase inhibitor, influence the antiproliferative action of esculetin (data not shown). This reveals that the antiproliferative effect of esculetin is not simply through the generation of the second messengers mentioned above. Furthermore, ROS are suggested to regulate the cell proliferative effect [21], and esculetin (100 μM) was examined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay to determine its free radical scavenging activity (about 83% scavenging activity). However, serum induced little generation of ROS (unpublished data). This result rules out the possibility that the free radical scavenging activity of esculetin mediates its antiproliferative action.

The activation of p42/44 MAPK and their downstream effectors, such as c-fos and c-jun, is well known to be a crucial event in cell proliferation [22]. These factors lead to transcription of the nuclear transcription factor, AP-1, which is a c-fos and c-jun heterodimer or a c-jun and c-jun homodimer complex that binds to the promoter region of many other early response genes [23]. Using western blot, RT–PCR, and EMSA analyses, we found that esculetin and PD98059 were able to inhibit serum-induced p42/44 MAPK phosphorylation, the expression of c-fos and c-jun mRNA, and the DNA binding activity of AP-1,

suggesting that esculetin, like PD98059, may inhibit cell proliferation through the regulation of the MAPK/c-fos/c-jun signaling pathway. Additionally, the growth factor-induced activation of MAPK also causes the translocation of NF-κB to the nucleus [6]; this early event, within the first 2 hr of treatment, regulates the cell proliferative effect in VSMCs. In the present study, we also determined the effect of esculetin and PD98059 on serum-induced NF-κB activation. Initially, we examined their effects on IκB-α phosphorylation and degradation as these two events are essential for the nuclear translocation and activation of NF-κB. We found that both esculetin and PD98059 significantly inhibited serum-induced IκB-α phosphorylation and its degradation, and therefore blocked the nuclear translocation of NF-κB. In an excellent study, Breuss *et al.* [24] used an adenoviral gene therapy approach to inhibit the central inflammatory mediator NF-κB by overexpression of its natural inhibitor IκB-α, and show data indicating that NF-κB is involved in post-angioplasty lumen narrowing. They also suggest that the use of specific and more potent inhibitors of NF-κB might be a useful therapeutic measure to improve the clinical outcome after balloon dilatation. This study might be considered as presenting direct evidence linking NF-κB to restenosis after angioplasty.

There are several lines of evidence suggesting that p42/44 MAPK plays a role in the regulation of NF-κB activity in VSMCs [6]. The blockade of the NF-κB-related cascade might be explained by the inhibition of MAPK-mediated signaling in the present work. It has been suggested that the MAPK-mediated signaling and immediate early genes, such as c-Fos, regulate the progression of the cell cycle induced by growth factors. Phuchareon and Tokuhisa [25] reported that in response to stimulation, c-fos expression is first increased and then cyclin D1 and cyclin E mRNA is induced in splenic B cells. In our present study, both esculetin and PD98059 significantly inhibited MAPK activation and c-fos mRNA expression. However, esculetin, but not PD98059 and U0126, profoundly reduced cyclin D1 expression, indicating that there exists an undefined modulator that is regulated differently by esculetin, PD98059, and U0126. It has been suggested recently that the PI 3-kinase-mediated pathway plays a key role in cyclin D1 expression and entry into the S phase of the cell cycle [26]. Furthermore, PI 3-kinase-activated Akt/PKB phosphorylation is sufficient to induce E2F transcriptional activity, and inhibition of PI 3-kinase inhibits the cyclin/cdk-mediated pathway [27]. It reveals the significance of PI 3-kinase/PKB-involved signaling in the regulation of the cell cycle machinery. Our data showed that esculetin, rather than PD98059, significantly inhibited serum-induced activation of PI 3-kinase, explaining their distinct regulation on cyclin D1 expression. Moreover, PD98059 exhibited a complete blockade of the MAPK pathway and then directed the serum stimulation to the PI 3-kinase-mediated pathway, resulting in an increase of Akt/PKB phosphorylation (Fig. 7).

It has been well recognized that Ras protein is a very early and upstream point of convergence for diverse extracellular signaling pathways [17]. In the present study, we determined the effect of esculetin on Ras activity based on two predominant reasons. One is that esculetin effectively inhibited serum-induced events, such as MAPK activation, immediate early gene expression, AP-1 and NF-κB activation, and PI 3-kinase activation. It revealed that the cardinal target of esculetin is before these events. The other is that esculetin and PD98059 regulated cyclin D1 expression differently, suggesting that esculetin acted on an event before MAPK-mediated signaling. As a result, our data demonstrated that esculetin markedly inhibited the serum-mediated increase of Ras activity.

Since esculetin effectively inhibited serum-mediated VSMC proliferation, the *in vivo* effect was then carried out to examine its therapeutic potential. The balloon injury model has been the most frequently used *in vivo* model to study restenosis. In addition, rats are regularly used because of the reproducibility of intimal hyperplasia within 2 weeks after balloon injury [28]. Our data showed that balloon-catheter inflation caused injury in the rat common carotid artery and intimal hyperplasia. Esculetin showed an *in vivo* potential to block the intimal thickening in the rat model.

In conclusion, we suggest that esculetin counters the proliferative effect of serum via the inhibition of Ras activity and downstream effectors, such as p42/44 MAPK, AP-1 and NF-κB transcription factors, c-*fos* and c-*jun* immediate early genes, and PI 3-kinase, thus inhibiting the cell cycle machinery. Esculetin also inhibits intimal hyperplasia after balloon vascular injury in the rat, indicating therapeutic potential for treating restenosis after arterial injury.

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