Inhibition of Cyclic Strain-Induced Endothelin-1 Secretion by Baicalein in Human Umbilical Vein Endothelial Cells

Hsi-Hsien Chen[1]
Hong-Jye Hong[2]
Yu-Hsiang Chou[3]
Tzu-Hurng Cheng[4]
Jin-Jer Chen[5, 6]
Heng Lin[6, 7]

Original Paper

Abstract

Baicalein is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Oriental medicine. Among its biological activities, baicalein has been reported to exhibit antioxidant effects. Endothelin-1 (ET-1) is a potent vasopressor synthesized by endothelial cells both in culture and *in vivo*. The aims of this study were to test the hypothesis that baicalein may alter strain-induced ET-1 secretion and to identify the putative underlying signaling pathways in endothelial cells. We show that baicalein inhibited strain-induced ET-1 secretion. Baicalein also inhibited strain-increased reactive oxygen species (ROS) formation and the extracellular signal-regulated kinases (ERK) phosphorylation. Using a reporter gene assay, baicalein and the antioxidant Trolox also attenuated the strain-stimulated activator protein-1 (AP-1) reporter activity. We conclude that baicalein inhibits strain-induced ET-1 gene expression, partially by interfering with the ERK pathway via attenuation of ROS formation. These results highlight the molecular pathways that may contribute to the beneficial effects of baicalein in the vascular system such as stroke prevention.

Key words
Endothelin-1 · baicalein · strain · endothelial cells · reactive oxygen species · extracellular signal-regulated kinases

Introduction

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Oriental medicine [1]. Baicalein is reported to act as a specific 12-lipoxygenase inhibitor and to possess many lipoxygenase-unrelated effects such as blocking calcium mobilization [2] and acting as an antioxidant [3]. Baicalein exhibits superior free radical scavenging activity among the flavonoid components of the herb [4] and has been shown to attenuate oxidative stress in cardiomyocytes [5]. Also, baicalein lowers blood pressure in renin-dependent hypertension, and the *in vivo* hypotensive effect may be partly attributed to its inhibition of lipoxygenase, resulting in reduced biosynthesis and release of arachidonic acid-derived vasoconstrictor products [6]. However, the mechanism of action for baicalein allowing for its possible use in the prevention and treatment of cardiovascular diseases remains unclear.

Endothelin-1 (ET-1) was originally isolated from a culture of porcine endothelial cells [7]. Recently, numerous studies have shown that oxidative stress, represented by reactive oxygen spe-

Affiliation
[1] Department of Medicine, Taipei Medical University Hospital, Taipei, Taiwan, R.O.C.
[2] School of Chinese Medicine, China Medical University, Taichung, Taiwan, R.O.C.
[3] Department of Nuclear Medicine, Taipei Tzu Chi General Hospital, Taipei County, Taiwan, R.O.C.
[4] Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, R.O.C.
[5] Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan, R.O.C.
[6] Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan, R.O.C.
[7] Graduate Institute of Pharmacology and Toxicology, Tzu Chi University, Hualien, Taiwan, R.O.C.

Correspondence
Heng Lin, Ph. D. · Department of Medicine · Taipei Medical University Hospital · Taipei · Taiwan · Republic of China · Phone: +886-2-7899135 · Fax: +886-2-7858594 · E-mail: linheng@ibms.sinica.edu.tw

Received September 12, 2005 · **Accepted** August 21, 2006

Bibliography
Planta Med 2006; 72: 1318–1321 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2006-951702 · Published online October 20, 2006
ISSN 0032-0943

cies (ROS), is capable of significantly altering vascular function [8]. Both ET-1 and oxidative stress have been the subjects of intense investigation within the cardiovascular field over the past decade [9]. Endothelial cells are constantly under the influence of mechanical forces, including cyclic strain, as a consequence of vessel contraction and relaxation [10]. We have demonstrated that intracellular ROS mediate cyclic strain-induced ET-1 expression via the Ras/Raf/extracellular signal-regulated kinases (ERK) signaling pathway [11]. However, no studies exist that address the interference of baicalein on ET-1 expression in vascular endothelial cells. The present study aimed at investigating the effect of baicalein on the strain-induced ET-1 expression and to identify signaling protein kinase cascades that may be responsible for the putative effect of baicalein.

Material and Methods

Materials

Imubind ET-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Amersham-Pharmacia (Amersham; Buckinghamshire, U.K.). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Serva Co. (Heidelberg, Germany). Hydrogen peroxide (H_2O_2) was purchased from Acros Organics (Pittsburgh, PA, USA). Trolox was purchased from Calbiochem (San Diego, CA, USA). Baicalein and all other chemicals of reagent grade were obtained from Sigma (St. Louis, MO, USA).

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as described previously [11]. All procedures involving human samples were conducted according to the Guidelines for Animal and Human Experimentation of the Taipei Medical University. The transformed human cell line, ECV304 (ATCC CRL-1998), was purchased from the American Type Culture Collection (Bethesda, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) before subculturing.

In vitro cyclic strain on cultured endothelial cells

Endothelial cells cultured on the flexible membrane base were subjected to cyclic strain produced by a computer-controlled application of sinusoidal negative pressure as described previously [11].

Measurement of ET-1 concentration

ET-1 levels were measured in culture medium using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Amersham-Pharmacia). Results were normalized to cellular protein content in all experiments and expressed as a percentage relative to the cells incubated with the vehicle.

Detection of intracellular ROS

Measurement of intracellular ROS formation in HUVECs was recorded by monitoring changes in diclorofluorescein (DCF) fluorescence as described previously [12].

Western blot analysis

Rabbit polyclonal anti-phospho-specific extracellular signal-regulated kinases (ERK) antibody was purchased from New England Biolabs (Beverly, MA, USA). Anti-ERK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blot analysis was performed as described previously [11].

Luciferase assay

ECV304 cells plated on 3-cm diameter culture dishes were transfected with the luciferase reporter construct possessing consensus AP-1 (AP-1-Luc) binding sites (Stratgene; La Jolla, CA, USA) by the calcium phosphate method as described previously [12]. After incubation for 24 hours in 2% serum DMEM, ECV304 were cultured under different treatments as indicated for 48 hours. ECV304 cells were assayed for luciferase activity with a luciferase reporter assay kit (Strategene). The firefly luciferase activities at AP-1 transcriptional activity were normalized for transfection efficiency to its respective β-galactosidase activity and expressed as relative activity to control.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using Student's t test and analysis of variance (ANOVA) followed by a Dunnett multiple comparison test using Prism version 3.00 for Windows (GraphPad Software; San Diego, CA, USA). P values of less than 0.05 were considered to be statistically significant.

Results and Discussion

HUVECs cultured on flexible membrane bases were subjected to deformation to produce an average strain of 20%. ET-1 released into the culture media was measured. HUVECs under cyclic strain for 24 hours increased their ET-1 secretion into the culture medium (Fig. **1**). Baicalein (1 – 100 μM) significantly inhibited strain-increased ET-1 secretion (Fig. **1**). These data indicate that baicalein inhibits strain-increased ET-1 secretion in endothelial cells.

We next examined whether baicalein prevents the strain-increased ROS formation. The addition of baicalein (1 – 100 μM) to

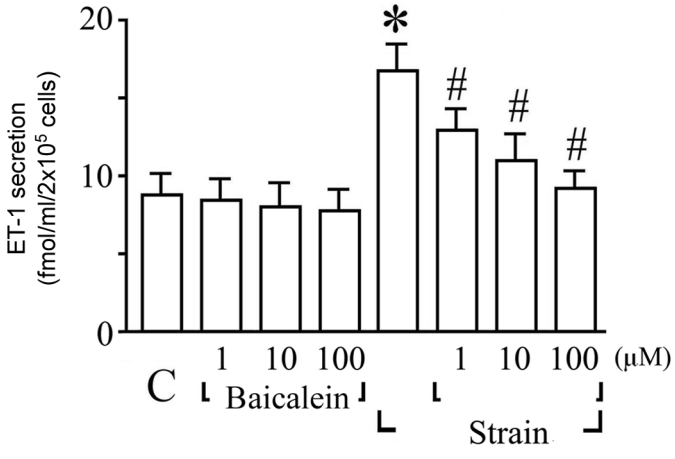

Fig. **1** Baicalein inhibits strain-induced ET-1 secretion. HUVECs were pretreated with baicalein (1 – 100 μM) 30 minutes prior to strain treatment. Results are presented as mean ± SEM (n = 6). * P < 0.05 vs. unstrained control cells. # P < 0.05 vs. strained cells (ANOVA).

cultured HUVECs significantly inhibited strain-induced ROS formation as measured after strain treatment for 1 hour (Fig. **2A**). The pretreatment with baicalein (100 μM) or the antioxidant Trolox (200 μM) in cultured HUVECs also significantly inhibited strain- or H_2O_2-induced ROS formation (Fig. **2B**). These findings clearly demonstrate that baicalein inhibits strain-increased intracellular ROS levels in endothelial cells.

We further investigated whether baicalein inhibits the ERK pathway in strain-treated endothelial cells. HUVECs pretreated with baicalein (1 – 100 μM) showed significantly decreased strain-induced ERK phosphorylation (Fig. **3A**). Moreover, HUVECs pretreated with baicalein (100 μM) or Trolox (200 μM) also showed significantly decreased strain- or H_2O_2-induced ERK phosphorylation (Fig. **3B**). These findings imply that baicalein inhibits strain-activated ERK signaling pathway via attenuation of ROS formation in endothelial cells.

We next evaluated the effect of baicalein on AP-1 activation, which is involved in ET-1 gene induction [13]. The effects of baicalein on strain-induced AP-1 functional activity were assessed in a reporter gene assay. Both baicalein (100 μM) and Trolox (200 μM) significantly attenuated strain- or H_2O_2-induced AP-1 reporter activation (Fig. **4**). These results indicate that baicalein inhibits strain-increased AP-1 transcriptional activation.

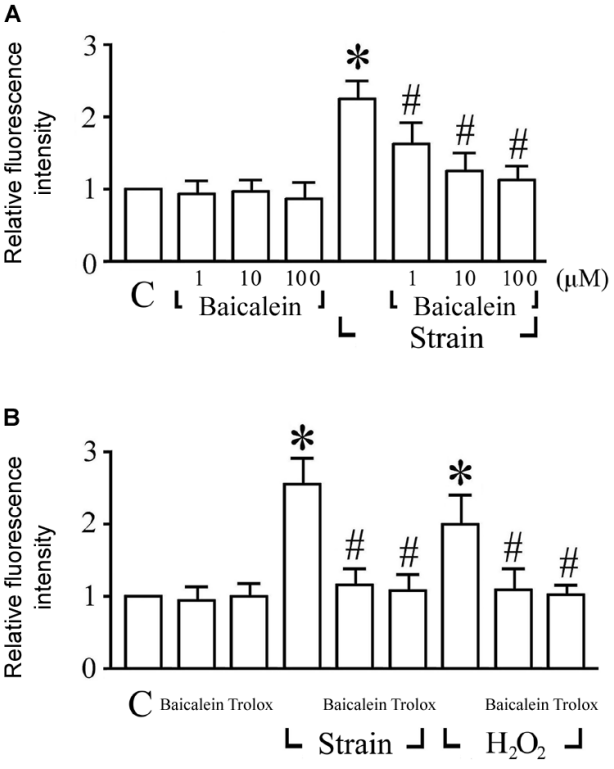

Fig. **2** Effects of baicalein on strain-increased ROS formation. **A** Effect of baicalein (1 – 100 μM) on strain-induced ROS generation. **B** Effect of baicalein (200 μM) and Trolox (200 μM) on HUVECs from either control (C) cells or cells treated with cyclic strain or H_2O_2 (25 μM) for one hour. Fluorescence intensities of cells are shown as relative intensity of experimental groups compared with untreated control cells. The results show mean ± SEM (n = 6). * P < 0.05 vs. control; # P < 0.05 vs. strain (or H_2O_2) treated cells (ANOVA).

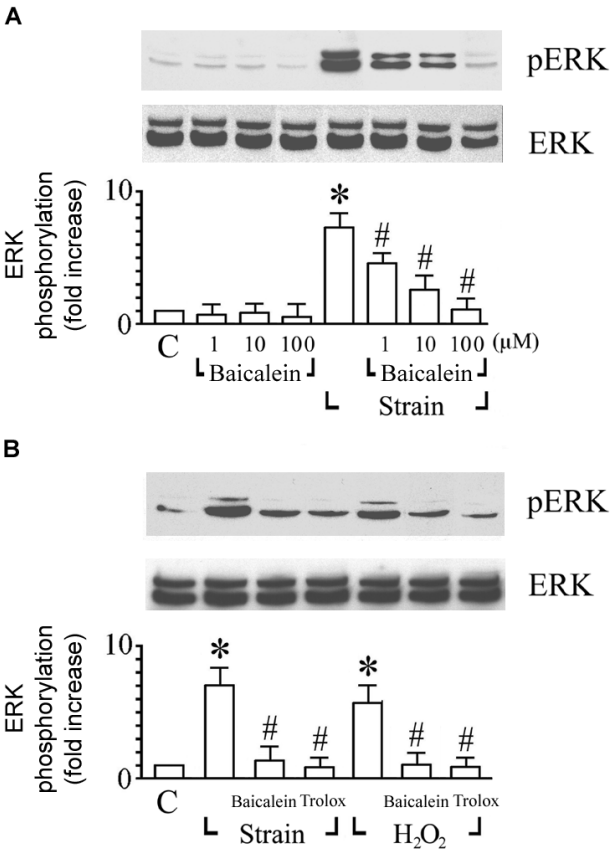

Fig. **3** Inhibitory effects of baicalein on strain-increased ERK phosphorylation. **A** Effect of baicalein (1 – 100 μM) on strain-activated ERK phosphorylation. **B** Effect of baicalein on strain- or H_2O2-induced phosphorylation of ERK. HUVECs were preincubated with either baicalein (100 μM) or Trolox (200 μM) for 30 minutes and stimulated with cyclic strain or H2O2 (25 μM) for 30 minutes. Data are represented as fold increase relative to control groups. The results show mean ± SEM (n = 6). * P < 0.05 vs. control; # P < 0.05 vs. strain (or H2O2) alone (ANOVA).

The major new finding of this work is that baicalein inhibits strain-induced ET-1 secretion in endothelial cells. This is supported by the observations that baicalein inhibits strain-induced ET-1 protein secretion in part via attenuation of ROS formation in endothelial cells. We have previously shown that cyclic-strain treatment of endothelial cells can induce intracellular ROS generation [12], [13]. Elevated ROS levels are involved in the release of ET-1 [12], [13], and this gene induction can be attenuated by antioxidant pretreatment of cells. The results of our present study demonstrate that baicalein reduced the strain-induced ROS generation in the endothelial cells. In particular, it has been demonstrated that activation of ERK is redox-sensitive and that suppression of ROS inhibits strain-induced ET-1 gene expression [11]. One possible explanation for the inhibitory effect of baicalein on strain-induced ET-1 gene expression may thus be its ability to attenuate ROS formation. In our previous study, we found that the activation of AP-1 is redox-sensitive and might play a key role in ET-1 gene induction [12], [13]. Our present results indicate that baicalein inhibits strain-induced AP-1 reporter activity. The inhibitory effect of baicalein on strain-induced AP-1 transcriptional activation suggested that the attenuation of strain-induced ROS by baicalein leads to inhibition of AP-1.

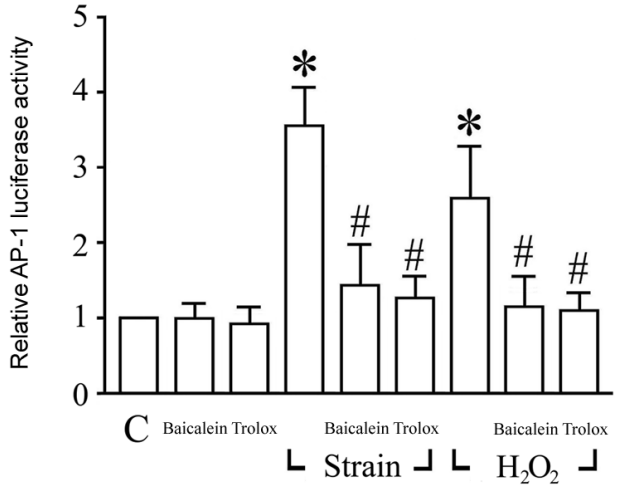

Fig. **4** Baicalein attenuates the strain-stimulated AP-1 reporter activity in endothelial cells. ECV304 cells, transfected with AP-1-Luc, were incubated for 24 hours with vehicle, baicalein (100 μM) or Trolox (200 μM) in the absence or presence of strain treatment or H_2O_2 (25 μM). Values are mean ± SEM of data for six experiments performed in triplicate. * $P < 0.05$ vs. control; # $P < 0.05$ vs. strain (or H_2O_2) alone (ANOVA).

From our results, the inhibitory effect of baicalein on strain-induced ET-1 expression is moderate. It had been reported that the H_2O_2 is produced during the auto-oxidation of baicalein [14]. These findings led us to speculate that the prooxidant effects of baicalein might be involved in its moderate inhibitory effect. However, similar pharmacological effects have also been reported for other flavonoids, such as resveratrol and tetramethylpyrazine in our previous studies [13], [15]. These compounds are believed to be beneficial for the prevention and treatment of cardiovascular diseases. In conclusion, the data obtained in the present study suggest that the baicalein-induced suppression of cyclic strain-induced ET-1 expression can be considered as one of the mechanisms responsible for the protective effect of baicalein in vascular vessels. These findings have highlighted the therapeutic potentials of using plant-derived baicalein for the treatment of arteriosclerosis and hypertension.

Acknowledgements

This work was supported, in part, by a National Science Council Grant (NSC 92-2314-B-038-051-), Topnotch Stroke Research Center Grant, Ministry of Education and Taipei Tzu Chi General Hospital Grant (TCRD-TPE-95-35), Taiwan, R.O.C.

References

[1] Shao ZH, Li CQ, Vanden Hoek TL, Becker LB, Schumacker PT, Wu JA et al. Extract from *Scutellaria baicalensis* Georgi attenuates oxidant stress in cardiomyocytes. J Mol Cell Cardiol 1999; 31: 1885 – 95
[2] Nyby MD, Sasaki M, Ideguchi Y, Wynne HE, Hori MT, Berger ME et al. Platelet lipoxygenase inhibitors attenuate thrombin- and thromboxane mimetic-induced intracellular calcium mobilization and platelet aggregation. J Pharmacol Exp Ther 1996; 278: 503 – 9
[3] Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radic Biol Med 1994; 16: 845 – 50
[4] Song LQ, Li Y, Zhang LN, Gao F, Cheng JH, Qi HW. [The inhibiting effect of niflumic acid on airway hyperresponsiveness in asthmatic mice]. Zhonghua Jie He He Hu Xi Za Zhi 2004; 27: 108 – 11
[5] Shao ZH, Vanden Hoek TL, Qin Y, Becker LB, Schumacker PT, Li CQ et al. Baicalein attenuates oxidant stress in cardiomyocytes. Am J Physiol Heart Circ Physiol 2002; 282: H999 – 1006
[6] Takizawa H, DelliPizzi AM, Nasjletti A. Prostaglandin I2 contributes to the vasodepressor effect of baicalein in hypertensive rats. Hypertension 1998; 31: 866 – 71
[7] Ahn K, Sisneros AM, Herman SB, Pan SM, Hupe D, Lee C et al. Novel selective quinazoline inhibitors of endothelin converting enzyme-1. Biochem Biophys Res Commun 1998; 243: 184 – 90
[8] Suzuki YJ, Nagase H, Nie K, Park AM. Redox control of growth factor signaling: recent advances in cardiovascular medicine. Antioxid Redox Signal 2005; 7: 829 – 34
[9] Pollock DM, Pollock JS. Endothelin and oxidative stress in the vascular system. Curr Vasc Pharmacol 2005; 3: 365 – 7
[10] Kakisis JD, Liapis CD, Sumpio BE. Effects of cyclic strain on vascular cells. Endothelium 2004; 11: 17 – 28
[11] Cheng TH, Shih NL, Chen SY, Loh SH, Cheng PY, Tsai CS et al. Reactive oxygen species mediate cyclic strain-induced endothelin-1 gene expression via Ras/Raf/extracellular signal-regulated kinase pathway in endothelial cells. J Mol Cell Cardiol 2001; 33: 1805 – 14
[12] Juan SH, Chen JJ, Chen CH, Lin H, Cheng CF, Liu JC et al. 17Beta-estradiol inhibits cyclic strain-induced endothelin-1 gene expression within vascular endothelial cells. Am J Physiol Heart Circ Physiol 2004; 287: H1254 – 61
[13] Liu JC, Chen JJ, Chan P, Cheng CF, Cheng TH. Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. Hypertension 2003; 42: 1198 – 205
[14] Miura YH, Tomita I, Watanabe T, Hirayama T, Fukui S. Active oxygens generation by flavonoids. Biol Pharm Bull 1998; 21: 93 – 6
[15] Bi WF, Yang HY, Liu JC, Cheng TH, Chen CH, Shih CM et al. Inhibition of cyclic strain-induced endothelin-1 secretion by tetramethylpyrazine. Clin Exp Pharmacol Physiol 2005; 32: 536 – 40