行政院國家科學委員會專題研究計畫　成果報告

Baicalein 於平滑肌細胞對血管收縮素所誘發內皮素基因表現的作用

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-039-050-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中國醫藥大學中醫學系

計畫主持人：洪宏杰
共同主持人：鄭志鴻

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 10 月 31 日

行政院國家科學委員會補助專題研究計畫 V 成果報告 □期中進度報告

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成果報告類型(依經費核定清單規定繳交)：V 精簡報告　□完整報告

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中　華　民　國　94　年　10　月　28　日

計畫中文摘要：

Baicalein 是黃芩，一種傳統中藥中所富含之天然抗氧化成份，在過去一系列實驗證實具有類似維他命 E (α-tocopherol)的抗氧化作用。於動物實驗上，證實可顯著的對抗高血壓以及減少心肌梗塞範圍及降低冠心疾病之發作。最近的研究報告亦顯示 baicalein 可明顯的降在血管平滑肌細胞的增生。然而，目前對於其在心血管系統的細胞作用與分子生物機轉仍不明確。

血管平滑肌細胞是血管系統的細胞，具有調節血管舒張或收縮及其他重要功能。近來實驗發現；血管收縮素(angiotenisn II; Ang II)可增加血管平滑肌細胞增生及內皮素-1(endothelin-1；ET-1)的基因表現。有此種作用與細胞內活性氧族群(reactive oxygen species; ROS)有關。實驗結果顯示血管收縮素所誘發血管平滑肌細胞增生及內皮素基因表現明顯為 baicalein 所抑制。同時 baicalein 明顯降低血管收縮素所誘發產生 ROS 以及 ERK 的磷酸化。此外，透過 chloramphenicol acetyltransferase (CAT) report gene assay 分析得知 AP-1 在 baicalein 對血管收縮素誘發 ET-1 的基因調控上扮演重要角色。總而言之，本實驗顯示 baicalein 透過抑制 ROS 以及 ERK 的磷酸化這個作用途徑進而降低其內皮素的釋出。這個機轉可能可以解釋 baicalein 對心血管的保護作用，並作為日後用藥的參考。

關鍵詞：內皮素、baicalein、 血管收縮素、 血管平滑肌細胞、活性氧族群。

英文摘要：

Baicalein is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Oriental medicine. Many studies have demonstrated that baicalein possess cardioprotective properties. However, mechanism of action by which baicalein in the prevention and treatment of cardiovascular diseases remains unclear. Endothelin-1 (ET-1) is a potent vasopressor synthesized by smooth muscle cells both in culture and in vivo. The aims of this study were to test the hypothesis that baicalein may alter AgII-induced ET-1 secretion and to identify the putative underlying signaling pathways in smooth muscle cells. We show that baicalein inhibits AgII-induced ET-1 secretion. Baicalein also inhibits AhII-increased reactive oxygen species (ROS) formation, and the extracellular signal-regulated kinases (ERK) phosphorylation. Using a reporter gene assay, baicalein and N-acetyl-cysteine also attenuated the AgII-stimulated activator protein-1 (AP-1) reporter activity. In summary, we demonstrate for the first time that baicalein inhibits AgII-induced ET-1 gene expression, partially by interfering with the ERK pathway via attenuation of ROS formation. Thus this study provides important new insights in the molecular pathways that may contribute to the proposed beneficial effects of baicalein in the vascular system.

Key words: endothelin-1, baicalein, angiotensin II, smooth muscle cells, reactive oxygen species.

前言、研究目的、文獻探討：

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Oriental medicine (Shao et al. 1999). Baicalein is reported to act as a specific 12-lipoxygenase inhibitor and also possess many lipoxygenase-unrelated effects such as blocking calcium mobilization (Nyby et al. 1996) and acting as an antioxidant (Hanasaki et al. 1994). Baicalein exhibits superior free radical scavenging activities among the flavonoid components of the herb (Song et al. 2004) and have been shown to attenuate oxidative stress in cardiomyocytes (Shao et al. 1999;Shao et al. 2002). 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 (Takizawa et al. 1998). However, mechanism of action by which baicalein in the prevention and treatment of cardiovascular diseases remains unclear.

Among the earliest indications of vascular dysfunction in atherosclerosis is an impaired regulation of vasomotion, representing disturbed homeostasis of vascular cells.(Gielen et al. 2001) Key regulators of vasomotor function are the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1). Among the endogenous mediators of cardiovascular disorders, ET-1, a 21-amino-acid peptide, is a primary antecedent in coronary heart disease.(Kuntz et al. 1999;Mundhenke et al. 1999;Schiffrin 2001;Schiffrin and Touyz 1998) Such effects are mediated by extremely potent vasopressors and mitogenic responses for ET-1 in the vasculature.(Hoffmann et al. 1998;Tonnessen et al. 1995) Results from preclinical studies in humans, as well as animals studies, showed that plasma ET-1 levels are consistently elevated in many spasm-related

cardiovascular diseases,(Hoffmann et al. 1998;Pernow and Wang 1997) and that blockers for ET receptors can substantially alleviate complications of such diseases.(Pernow and Wang 1997;Webb et al. 1998)

Recently, numerous studies have shown that oxidative stress, represented by reactive oxygen species (ROS), is capable of significantly altering vascular function.(Drexler 1997;Witztum 1994;Zafari et al. 1999) The present study was aimed to investigate the effect of baicalein on the AgII-induced ET-1 expression and to identify signaling protein kinase cascades that may be responsible for the putative effect of baicalein.

研究方法

Materials

Imubind ET-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Amersham-Pharmacia (Amersham, 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, U.S.A.). Baicalein, N-acetyl-cysteine (NAC), and all other chemicals of reagent grade, were obtained from Sigma (St. Louis, MO, U.S.A.).

Measurement of ET-1 Concentration

ET-1 levels were measured in culture medium using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Amersham-Pharmacia, Amersham, U.K.). 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.(Cheng et al. 2001)

Western Blot Analysis

Rabbit polyclonal anti-phospho-specific extracellular signal-regulated kinases (ERK) antibody was purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-ERK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Western blot analysis was performed as described previously.(Liu et al. 2003)

Luciferase Assay

Smooth muscle cells plated on 3-cm diameter culture dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) binding sites (Stratgene, La Jolla, CA, U.S.A.) by the calcium phosphate method as described previously.(Liu et al. 2003) After incubation for 24 hours in 2% serum DMEM, ECV304 were cultured under different treatments as indicated for 48 hours. ECV304 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 $\pm$ 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 less than 0.05 were considered to be statistically significant.

結果與討論：

Effect of baicalein on AgII-induced ET-1 secretion in smooth muscle cells

Smooth muscle cells treated with AgII (100 nM) for 24 hours increased their ET-1 secretion into the culture medium (Figure 1) 2-fold compared with untreated smooth muscle cells. We then examined the effect of baicalein on AgII-increased ET-1 secretion. As shown in Figure 1, treatment with AgII for 24 hours increased ET-1 secretion. Baicalein (1-100 μM) significantly inhibited AgII-increased ET-1 secretion (Figure 1). These data indicate that baicalein inhibits AgII-increased ET-1 secretion in smooth muscle cells.

Baicalein inhibits AgII-increased ROS formation

We examined whether baicalein prevents the AgII-increased ROS formation. Smooth muscle cells were treated with baicalein (1-100 μM) in the absence or presence of AgII treatment. The addition of baicalein (1-100 μM) to cultured smooth muscle cells significantly inhibited AgII-induced ROS formation (Figure 2A, B). The pretreatment of baicalein (100 μM) or NAC (10 mM) to cultured HUVECs also significantly inhibited AgII- or H_2O_2-induced ROS formation (Figure 2C). These findings clearly demonstrate that baicalein inhibits AgII-increased intracellular ROS levels in smooth muscle cells.

Baicalein inhibits AgII–activated ERK phosphorylation in smooth muscle cells

To gain insight into the mechanism of action of baicalein, we thus examined whether baicalein affects intracellular protein kinase signaling pathways. Given that the ERK signaling pathway is involved in AgII-induced ET-1 expression,(Liu et al. 2003) we further investigated whether baicalein inhibits the ERK pathway in AgII-treated smooth muscle cells. We examined

the phosphorylation of ERK in smooth muscle cells exposed to baicalein (1-100 μM) in the absence or presence of AgII treatment. As shown in Figure 3, smooth muscle cells exposure to strain treatment for 30 minutes rapidly activated phosphorylation of ERK. However, smooth muscle cells pretreated with baicalein (100 μM) showed significantly decreased strain-induced ERK phosphorylation. Moreover, smooth muscle cells treated with H_2O_2 (25 μM) showed increased ERK phosphorylation (Figure 3). Smooth muscle cells pretreated with baicalein (100 μM) or NAC (10 mM) showed significantly decreased AgII- or H_2O_2-induced ERK phosphorylation. These findings imply that baicalein inhibits AgII-activated ERK signaling pathway via attenuation of ROS formation in smooth muscle cells.

Baicalein inhibits AgII–increased AP-1 transcriptional activity in smooth muscle cells

We next evaluated the effect of baicalein on AP-1 activation, which is involved in ET-1 gene induction.(Liu et al. 2003) The effects of baicalein on AgII-induced AP-1 functional activity were assessed in a reporter gene assay. Either baicalein (10 μM) or NAC (10 mM) significantly attenuated strain- or H_2O_2–induced AP-1 reporter activation (Figure 4). These results indicate that baicalein inhibits strain-increased AP-1 transcriptional activation.

DISCUSSION

The major new finding of this work is that baicalein inhibits AgII-induced ET-1 secretion in smooth muscle cells. It is supported by the observations that baicalein inhibits AgII-induced ET-1 protein secretion in part via attenuation of ROS formation in endothelial cells. Recent studies provide evidence that ROS may act as second messengers in cells exposed to various stimuli.(Liu et al. 2003) Elevated ROS levels are involved in the release of ET-1,(Liu et al. 2003) and this

gene induction can be attenuated by antioxidant pretreatment of cells.

Baicalein exhibits superior free radical scavenging activities among the flavonoid components of the herb (Song et al. 2004) and have been shown to attenuate oxidative stress in cardiomyocytes (Shao et al. 1999;Shao et al. 2002). The results of our present study further demonstrated that baicalein reduced the AgII-induced ROS generation in the smooth muscle cells. In particular, it has been demonstrated that activation of ERK is redox-sensitive(Cheng et al. 2001;Sano et al. 2001;Tanaka et al. 2001) and that suppression of ROS inhibits strain-induced ET-1 gene expression.(Liu et al. 2003) One possible explanation for the inhibitory effect of baicalein on AgII-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.(Cheng et al. 2003;Juan et al. 2004;Liu et al. 2003) Our present results indicate that baicalein inhibits AgII-induced AP-1 reporter activity. The inhibitory effect of the baicalein on strain–induced AP-1 transcriptional activation suggested that the attenuation of strain-induced ROS by baicalein leads to inhibition of AP-1.

In conclusion, the data obtained in the present study suggest that the baicalein-induced suppression of AgII-induced ET-1 expression can be considered as one of the mechanisms responsible for the protective effect of baicalein in vascular vessels. The effects of baicalein on smooth muscle cells observed in the present study, i.e., inhibition of ET-1 secretion, suppression of ROS formation, and inhibition of ERK phosphorylation, all are compatible with its putative vasoprotective effect. These findings have highlighted the therapeutic potentials of using plant-derived baicalein for the treatment of arteriosclerosis and hypertension.

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FIGURE LEGENDS

Figure 1. Baicalein inhibits AgII-induced ET-1 secretion. Smooth muscle cells were pretreated with baicalein (1-100 μM) 30 minutes prior to AgII (100 nM) treatment. After treatment with AgII for 24 hours, the culture media were collected and the concentrations analyzed by enzyme immunoassay. Results are presented as mean ± SEM (n=6). *P < 0.05 vs. unstrained control cells. # P <0.05 vs. strained cells (ANOVA).

Figure 2. Effects of baicalein on AgII-increased ROS formation. (A) Effect of baicalein (1-100 μM) on AgII-induced ROS generation. AgII-increased intracellular ROS levels were revealed by fluorescent intensities of DCF. (B) Smooth muscle cells from either control (C; column 1) or treated with AgII (100 nM) or H_2O_2 (25 μM) in the presence of 100 μM baicalein, 10 mM NAC for 1 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).

Figure 3. Inhibitory effects of baicalein on AgII-increased ERK phosphorylation. (A) Effect of baicalein (1-100 μM) on AgII-activated ERK phosphorylation. (B) Effect of baicalein on AgII- or H_2O_2-induced phosphorylation of ERK. Smooth muscle cells were preincubated with either the baicalein (100 μM), or NAC (10 mM) for 30 minutes and stimulated with AgII or H_2O_2 (25 μM) for 30 minutes. Phosphorylation of ERK was detected by Western blotting using anti-phospho-ERK antibody. Both baicalein and NAC inhibited strain-induced activation of ERK. Phosphorylation of ERK was detected, and densitometric analyses were performed. 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. AgII (or H_2O_2) alone (ANOVA).

Figure 4. Baicalein attenuates the strain-stimulated AP-1 reporter activity in smooth muscle cells, transfected with AP-1-Luc, were incubated for 24 hours with either no drug, 100 µM baicalein, or 10 mM NAC in the absence or presence of AgII treatment or H_2O_2 (25 µM). Values are mean ± SEM of data for six experiments performed in triplicate. *P< 0.05 vs. untreated. #P< 0.05 vs. AgII (or H_2O_2) alone (ANOVA).

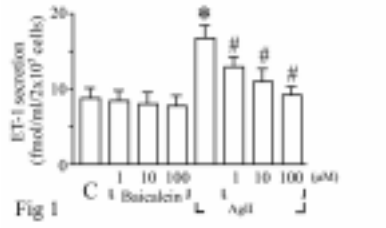

Fig 1

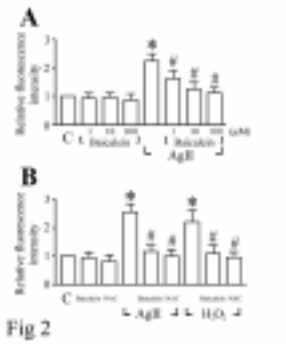

Fig 2

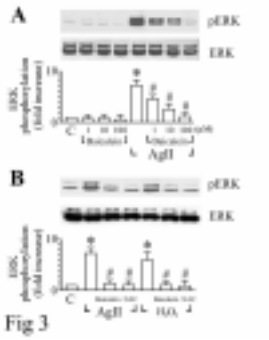

Fig 3

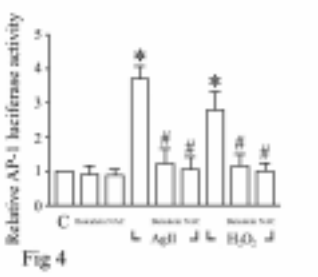

Fig 4