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Baicalin Induces Differential Expression of Cytochrome *C* Oxidase in Human Lung H441 Cell

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In a previous study, we evaluated the effect of baicalin on the expression of SP-A (surfactant protein A), which was developmentally regulated in an alveolar type II cell, H441. SP-A is encoded by two similar genes, *SP-A1* and *SP-A2*, in humans. The maximal induction of SP-A1 gene of H441 occurred at treating 150 nM of baicalin for 48 h. In the present study, cDNA subtraction analysis is performed to examine the differential expression in H441 cell upon baicalin treatment with a view to investigating the regulatory mechanism. The mRNA of H441 cell incubated with 150 nM baicalin for 48 h was compared to that of blank control. Two PCR products were obtained through subtractive cDNA amplification. A product encoding cytochrome *c* oxidase was demonstrated to be a differential signal by RT-PCR analysis, and the other was a false positive. The induction of cytochrome *c* oxidase might increase ATP level in cell, and consequently elevates cAMP, which upregulates surfactant synthesis and secretion.

KEYWORDS: Baicalin; cytochrome *c* oxidase; cDNA subtraction

INTRODUCTION

Baicalin, 5,6,7-trihydroxyflavone-7-β-D-glucuronide, is a major component in the root of *Scutellaria baicalensis* Georgi (Scutellariae Radix). Recent studies have shown that baicalin inhibits the proliferation of prostate cancer cell LNCaP via apoptosis and arrests cell cycle at the G_1 stage (*1*, *2*). This compound also has antibacterial activity as it inhibits the growth of *Pseudomonus aeruginosa* and *Staphylococcus aureus* (*3*). In addition, baicalin inhibited the infection and replication of human immunodeficiency virus (HIV) and is therefore a potential anti-HIV agent (*4*). The compound is an ingredient in a famous traditional Chinese prescription, sho-saiko-to. The growth inhibitions of the human hepatoma cell line, KIM-1, between treatments of sho-saiko-to and its ingredients were compared. The highest inhibition was observed in the sho-saiko-to treatment, and baicalin was the next potent. The other ingredients showed no marked inhibition (*5*). Additionally, baicalin has also been reported to exhibit antioxidative activity through inhibiting lipid peroxidation in rat brain and kidney homogenates (*6*). Scutellariae Radix is extensively used in combination with other Chinese medicines in the prescription for preventing miscarriage in China. The aim of our study is to evaluate the activity of baicalin on the gene expression of human lung cell.

Respiration distress syndrome is a major cause of morbidity and mortality in preterm neonates. It results from insufficient production of pulmonary surfactant by the premature lung. Maternal glucocorticoid treatment decreased the incidence of death and RDS by 50% in clinical experiments (*7*). However, glucocorticoids were recently reported to have adverse effects on the growth and development of the immature brain *in vivo* (*8*, *9*). In a previous study, we evaluated baicalin on the maturation of H441, a type II lung cell. The results showed that the expression of lung-specific SP-A gene was positively affected by baicalin in both dose-dependent and time-course manners. In the present study, a cDNA subtraction technique was employed to compare gene expression between the H441 cell line treated with baicalin and control. Schraml et al. (*10*) obtained seventeen differential display cDNA clones, which were expressed in normal lung tissue but decreased or absent in the corresponding tumor cell, using a magnet-assisted subtraction technique. Shinohara et al. (*11*) screened to find an NAD^+-dependent isocitrate dehydrogenase gene with expression in the brown adipose tissue of rat but not in the white adipose tissue, by a PCR-select cDNA subtraction approach. The same analysis was also performed in this study to observe the differential display gene expression between H441 lung cancer cells treated with baicalin and the control. We obtained a subtractive cDNA clone, cytochrome *c* oxidase, significantly expressed in the treated cell, but not in the control cell. The enzyme is responsible for the redox reaction in the electron transport chain and thereby ATP generation in the cell, which provides a logical explanation for its positive regulation of surfactant synthesis and secretion in lung development.

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MATERIALS AND METHODS

Cell Culture. Lung adenocarcinoma cells NCI−H441 were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum in an atmosphere containing 5% CO_2 at 37 °C. The cells were grown to confluence in 100-mm plastic tissue culture dishes, then cultured in either control medium or the same medium supplemented with 150 nM of baicalin (Wako Pure Chemical Industries, Japan) for 48 h.

PCR-Select cDNA Subtraction. Total RNA was extracted from H441 using RNAzol B reagent (Protech, Inc., USA). Messenger RNA was purified by a QIAGEN Oligotex mRNA purification kit (QIAGEN Inc., USA). The extracted mRNA was dissolved in a commercial RNA storage solution (Ambion Inc., USA) and stored at −80 °C. Messenger RNA quantity was determined by absorbance at 260 nm. For isolation of cDNA clone predominantly expressed in the treated cell but not in the control cell, mRNAs of the treated cell and the control cell were used as "tester" and "driver", respectively. The subtraction of driver RNA from tester RNA was carried out according to the user manual of the PCR-select cDNA subtraction kit (Clonetech, Inc., USA).

Cloning and Sequencing. The subtracted DNAs were subcloned on the pGEM-T vector (Promega, USA) and transformed into the competent cell JM109. The sequencing reactions of the subcloned products were carried out using the ABI PRISM Dye Terminator Cycle Sequencing Automatic System according to the manufacturer's protocol.

Reverse Transcriptase-PCR (RT-PCR) analysis. RT-PCR was performed to confirm the subtracted products of the selective PCR technique using a one-step Fast-Run M-MLV RT-PCR kit (Protech, Inc., USA). The cytochrome *c* oxidase forward primer is 5′CAGGAAATAGAAACCGTCTGAACTATCCTG3′, and the reverse primer is 5′CTGTGGTTTGCTCCACAGATTTCAGTGCAT3′. The Cu/Zn SOD forward primer, 5′AGTGCAGGGCATCATCAATTTCGAGCAG3′, and the reverse primer, 5′CAGGATACATTTCTACAGCTAGCAGGAT3′, were designed to amplify a 400 bp and a 439 bp PCR product, respectively. The β- actin forward primer, 5′GTGGGGCGCCCCAGGCACCA3′, and reverse primer, 5′CTCCTTAATGTCACGCACGATTTC3′, were also synthesized to amplify a 530 bp fragment for internal control. The reactions were first performed at 50 °C for 30 min, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and 30 s, and extension at 74 °C for 2 min. The products of PCR were separated on 2% agarose gel by electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) and stained with ethidium bromide.

RESULTS

PCR-Select cDNA Subtraction and Sequencing. Previous studies have shown that maximal SP-A expression in H441 cell culture was induced by 150 nM baicalin treatment for 48 h. Messenger RNA was extracted from the same cell for differential display analysis in this study. A modified DDRT-PCR method was adopted to obtain the differential expression gene and reduce false products. In the PCR-select cDNA subtraction procedure, one set of adaptors was ligated to the ends of the subtracted cDNA to create two unique annealing sites by specific primers, which prevents the generation of nonspecific PCR products. Two distinct bands, approximately 350 and 480 bp in length, were differentially produced in the PCR of the subtracted sample, but not in the blank sample. These bands were isolated from agarose gel and cloned into pGEM-T vector sequencing. The partial fragments of the two subtracted products with sizes of 294 and 275 bp were resolved. Homology analysis for these two sequences was performed by searching the GenBank. The 294-bp sequence shares a 96% homology with Cu/Zn SOD (**Figure 1A**) and differs from the published sequence by twelve nucleotides. The 275-bp sequence shares a 99.6% homology with both cytochrome *c* oxidase and the transducin-like enhancer of split 1. There is only one mismatch with the two known sequences (**Figure 1B**).

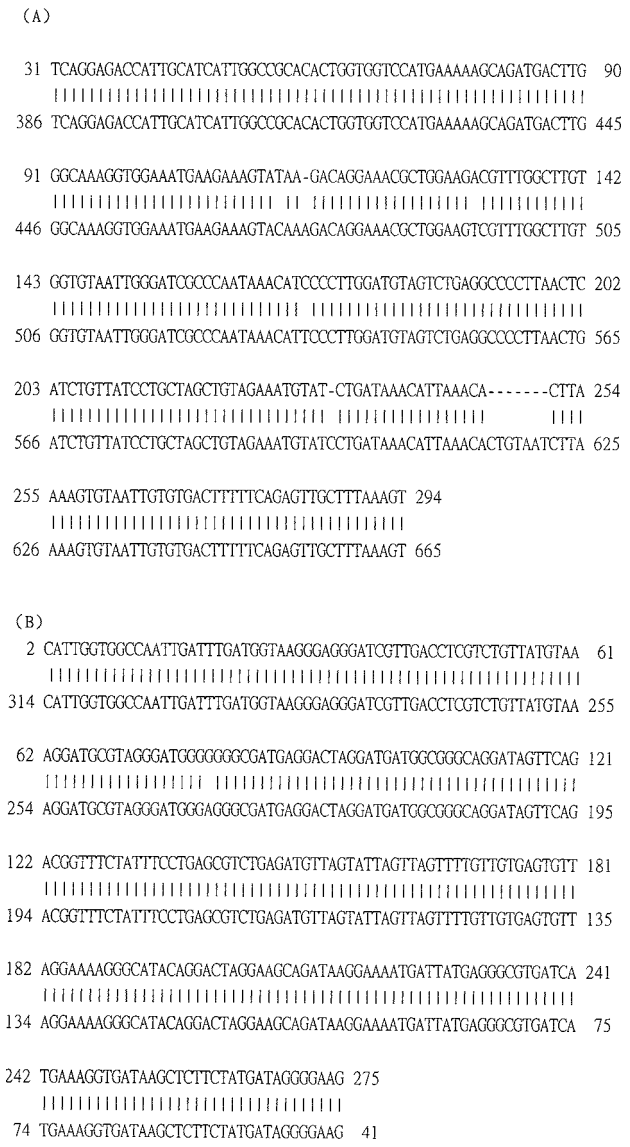

Figure 1. Comparison of nucleotide sequences (A) between copper–zinc superoxide dismutase of human and the 294-bp cDNA, and (B) between cytochrome *c* oxidase of human and the 275-bp cDNA.

Quantitative RT-PCR Analysis. To confirm the subtracted cDNAs were really a differential display between the control and the baicalin treatment, two sets of specific primers were designed according to the sequences of these two cDNAs for quantitative RT-PCR analysis. β-actin was used as an internal control for calibration. The results of RT-PCR analysis showed that no marked difference of Cu/Zn SOD expression was found between the control and the treatment. However, a consistent 2-fold increase in expression was revealed in the cytochrome *c* oxidase PCR product upon baicalin treatment (**Figure 2**).

DISCUSSION

In the present study, we focused on the effect of baicalin on the gene expression of the H441 lung cell culture by PCR select cDNA subtraction. We found that cytochrome *c* oxidase was significantly increased in the treated cells. The enzyme is responsible for catalyzing electron transportation in cell respiration, resulting in ATP generation and free radical removal.

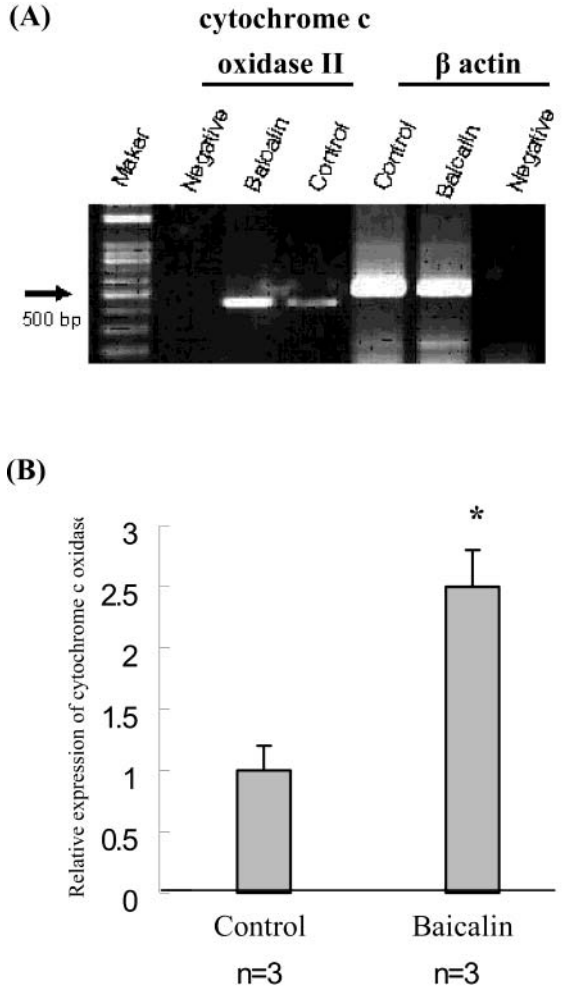

Figure 2. (A) RT-PCR analysis for cytochrome *c* oxidase and β-actin in human cancer cell line H441. (B) Relative expression of cytochrome *c* oxidase in H441 cell induced by baicalin. The H441 cell was cultured in either control RPMI 1640 medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C for 48 h or the same medium supplemented with 150 nM of baicalin.

Pulmonary surfactant is a complex mixture of glycerophospholipids, cholesterol, and proteins. The surfactant contains four unique proteins, namely surfactant protein A (SP-A), SP−B, SP−C, and SP-D, which are synthesized by the alveolar type II cell. Surfactant not only reduces the surface tension at the alveolar air−liquid interface but is also involved in host defense and inflammatory processes of the lung (*12*). Surfactant secretion is regulated by multiple factors. Rooney (*13*) reported that it is mediated by at least three distinct signaling mechanisms: activation of adenylate cyclase, activation of protein kinase, and activation of Ca^{+}-calmodulin dependent protein kinase. ATP and TPA are thought to be more efficient agonists that stimulate the surfactant secretion of normal type II cell four times more than the basal line. However, the other kind of agonist, β-adrenergic agonists, cholera toxin, and calcium ionophores, produced only a 2-fold surfactant secretion (*13*, *14*). ATP activates adenylate cyclase activity to increase formation of cAMP and subsequently activate the activity of protein kinase A (PKA) (*15*). In another mechanism, ATP activates protein kinase C (PKC) activity through activation of phospholipase C (PLC-β3) and formation of diacylglycerol (DAG) (*16*). Li et al. (*17*) demonstrated that cAMP increased the binding and transcriptional activities of thyroid transcription factor-1 (TTF-1), a trans-acting element of SP-A. The promoter activity

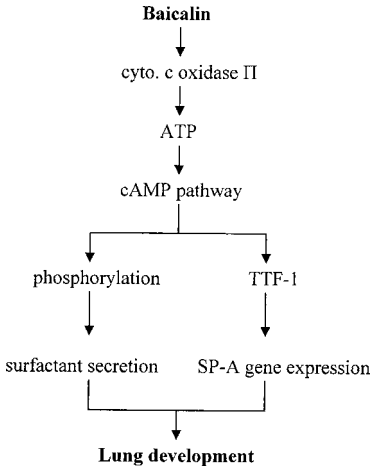

Figure 3. A proposed scheme of baicalin on the regulation of lung development.

of SP-A2 was induced by binding of TTF-1 to three binding elements (TBEs) flanking the 5′ end of the gene. They suggest that the PKA-induced increase of TTF-1 phosphorylation and TBE binding activity mediates the cAMP-induced expression of SP-A in A549 lung cancer cell. In this study, we obtained an ATP generation-related enzyme, cytochrome *c* oxidase, which was differentially expressed in the H441 lung cancer cell treated by baicalin. The elevation of ATP level will stimulate the increase of cAMP in rat type II cell (*18*). This finding may provide an explanation for the role baicalin plays in lung development (**Figure 3**) as an inducer of surfactant secretion and SP-A expression. On the other hand, baicalin, a strong antioxidant in Scutellariae Radix, exerts a free radical scavenging function by inhibiting the activity of xanthine oxidase (*19*). Several other components of the tocolytic Chinese prescription also demonstrated antioxidative activity, so it may be worth investigating the effect of the free radical on the development of the lung cell. In the present study, the expression of cytochrome *c* oxidase elevated by baicalin resulted in decreasing the level of free radical in the cell, which may change the gene expressions related to lung maturation. Ayad and Wong (*20*) reported that SP-A expression was reduced by 30% when H441 cell culture was treated with a nitric oxide (NO) free radical donor, S-nitroso-*N*-acetyl penicillamine, at 3.0 mM for 24 h. They demonstrated that NO exerted a negative effect on the surfactant function. We will further investigate the effect of hydroxyl radical on the synthesis of phospholipids and surfactant proteins in lung cell in a future study.

In an attempt to modernize traditional Chinese medicine, we have focused our attention on the biological function of baicalin, which targets fetal lung maturation. The model established may be applied to other single components or complex active compounds in Chinese medicine. Our work showed that baicalin differentially expresses an electron transport flow enzyme, cytochrome *c* oxidase. The enzyme activation may be due to in ATP elevation and free radical removal in the cell, which may be some of the roles played by baicalin. The study of how baicalin affects the phospholipid synthesis of lung in fetal rat is being evaluated.

ABBREVIATIONS USED

cAMP, cyclic adenosine monophospate; Cu/Zn SOD, copper−zinc superoxide dismutase; DDRT-PCR, differential display reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription- polymerase chain reaction; SP, surfactant

protein; TPA, tetradecanoyl phorbol actate; TTF-1, thyroid transcription factor-1

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