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Strong antiproliferative effects of baicalein in cultured rat hepatic stellate cells

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Abstract

Recently, antifibrogenetic effects of Sho-saiko-to, a traditional herbal medicine in Japan, have been shown in experimental hepatic fibrosis, and flavonoids in Sho-saiko-to are suspected as active ingredients. Thus, we evaluated the effects of baicalein, a major flavonoid in Sho-saiko-to, on proliferation and protein synthesis in cultured rat hepatic stellate cells. Baicalein decreased [^{3}H]thymidine incorporation in cells stimulated with platelet-derived growth factor-B subunit homodimer (PDGF-BB) in a concentration-dependent manner (approximate $ED_{50} < 10$ μM, $P < 0.0001$), and the decrease observed with 10 μM baicalein was greater than those observed with 5 μM retinol or 500 μM 3-isobutyl-1-methylxanthine (IBMX). Baicalein consistently decreased [^{3}H]thymidine incorporation and cell number in cells stimulated with fetal calf serum ($ED_{50} < 10$ μM, $P < 0.0001$), and moderately suppressed [^{3}H]leucine and [^{3}H]proline incorporation ($P < 0.0001$). These results demonstrate the strong antiproliferative effect of baicalein in hepatic stellate cells, showing the possibility of baicalein as an antifibrogenetic drug for hepatic fibrosis.

Keywords: Sho-saiko-to; Flavonoid; Baicalein; Proliferation; Hepatic stellate cell; Hepatic fibrosis

1. Introduction

Scutellariae radix, the root of *Scutellaria baicalensis* Georgi, is a conventional herbal medicine, and is widely used for traditional herbal preparations in Japan and China. In Japan, Sho-saiko-to, a traditional herbal preparation containing Scutellariae radix, is commonly used to treat patients with chronic hepatitis as an approved prescription drug. In addition to the antiinflammatory effect on chronic hepatitis (Hirayama et al., 1989; Kakumu et al., 1991), Sho-saiko-to has a beneficial effect to prevent the development of hepatocellular carcinoma in patients with liver cirrhosis (Oka et al., 1995). Recently, the antifibrogenetic effects of Sho-saiko-to have been demonstrated in experimental hepatic fibrosis in animals (Amagaya et al., 1989; Miyamura et al., 1998; Sakaida et al., 1998; Shimizu et al., 1999). Moreover, the suppressive effects of Sho-saiko-to on proliferation and collagen synthesis were shown in cultured hepatic stellate cells (also called hepatic lipocytes, Ito cells or fat-storing cells) (Kayano et al., 1998), which play a central role in the pathogenesis of hepatic fibrosis in chronic liver diseases (Friedman, 1993; Hautekeete and Geerts, 1997). In their study, Sho-saiko-to arrested hepatic stellate cells at G0/G1 phase and inhibited cell proliferation. Similar effects of Sho-saiko-to were observed in human hepatocellular carcinoma and cholangiocarcinoma cell lines (Yano et al., 1994). In these in vitro studies, flavonoids derived from Scutellariae radix in Sho-saiko-to were highly suspected as active ingredients for these effects (Yano et al., 1994; Kayano et al., 1998).

Scutellariae radix contains four major flavonoids with the same basic chemical structure of 2-phenyl-4*H*-1-benzopyran-4-one, i.e., baicalein, baicalin, wogonin and wogonoside (Sekiya and Okuda, 1982; Huang et al., 1994). Baicalein and wogonin have very close chemical structures which are designated as 5,6,7-trihydroxy-2-phenyl-4*H*-1-benzopyran-4-one and 5,7-dihydroxy-8-methoxy-2-phenyl-4*H*-1-benzopyran-4-one. Baicalin and wogonoside are the glucuronic acid glycosides of baicalein and wogonin, respectively. In cultured human gingival fibroblasts, these

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flavonoids augment cellular activity and induce collagen and total protein synthesis (Chung et al., 1995). In contrast, these flavonoids have antiproliferative effects on aortic smooth muscle cells (Huang et al., 1994) and human hepatocellular carcinoma cell lines in in vitro studies (Motoo and Sawabu, 1994; Matsuzaki et al., 1996), and baicalein and baicalin showed potent antiproliferative effects in these studies. In a previous study, only a high concentration of baicalein (approximately 185 μM) showed a suppressive effect on proliferation in cultured hepatic stellate cells according to their brief statement of unshown data (Kayano et al., 1998). Moreover, a very recent study indicated that the antioxidative activities of Scutellariae radix-derived flavonoids in Sho-saiko-to are also importantly involved in the antifibrogenetic effect of this drug in experimental hepatic fibrosis through the inhibition of lipid peroxidation in hepatocytes and hepatic stellate cells (Shimizu et al., 1999). Thus, to elucidate the mechanism of the antifibrogenetic effect of Sho-saiko-to, it is necessary to carefully clarify the effects of the flavonoids on proliferation and protein synthesis in hepatic stellate cells.

Therefore, in the present report, we studied the effects of baicalein, a major bioactive flavonoid derived from Scutellariae radix in Sho-saiko-to, on proliferation and protein synthesis in cultured rat hepatic stellate cells.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium/nutrient mixture F-12; 1:1 mixture (DMEM/F12), Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), penicillin–streptomycin solution and 0.25% trypsin–EDTA solution were purchased from Gibco BRL (Grand Island, NY). Fetal calf serum was purchased from HyClone Laboratories (Logan, UT). Baicalein, 3-isobutyl-1-methylxanthine (IBMX), retinol acetate, rat platelet-derived growth factor-B subunit homodimer (PDGF-BB), bovine serum albumin, sudan IV, Mayer's hematoxylin solution and all tissue culture ware were purchased from Sigma (St. Louis, MO). Monoclonal antibodies to smooth muscle α-actin and vimentin were purchased from Boehringer Mannheim (Indianapolis, IN). Labelled streptavidin biotin method kit (DAKO LSAB2) and substrate-chromogen reagent kit (DAKO AEC Substrate System) were purchased from DAKO (Carpinteria, CA). [Methyl-^{3}H]thymidine (20 Ci/mmol), L-[4,5-^{3}H(N)]leucine (50 Ci/mmol) and L-[2,3,4,5-^{3}H]proline (80 Ci/mmol) were purchased from NEN Life Science (Boston, MA).

2.2. Isolation of rat hepatic stellate cells by explant culture

Livers were obtained from 170 to 200 g male Sprague–Dawley rats (Charles River, Wilmington, MA) anesthetized with pentobarbital. After PBS was flushed through the portal vein in situ, the liver capsule and large vessels were removed, and liver tissue was minced and plated with DMEM/F12 containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 20% fetal calf serum in 75 cm^2 tissue culture flasks. Flasks were incubated under standard tissue culture conditions (37°C, 5% CO_2/95% air and 98% humidity), and the medium was changed every 3 days. The first outgrowth of cells with clear nuclei and multiple cytoplasmic droplets was observed within 5 days (Fig. 1A), followed by rapid and dominant outgrowths of cells with the same morphology in every flask. The lipid nature of the cytoplasmic droplets was confirmed by positive lipid staining with sudan IV in the outgrowth-cells of several primary culture flasks on the 10th day of primary culture (Fig. 1B). The cytoplasmic lipid droplets were clearly identified by phase-contrast microscopy. On the 14th day, cell colonies in which cytoplasmic lipid droplets could not be observed by phase-contrast microscopy were removed with a cell scraper in the remaining primary culture flasks. Approximately 70% of the growth surface in the primary culture flasks was covered with the cells in the following 2 to 3 weeks. The identity of the remaining cells was further confirmed by positive immunoperoxidase stainings with monoclonal antibodies to smooth muscle α-actin (Fig. 1C) and vimentin (Fig. 1D) in first passaged cells by means of the labelled streptavidin–biotin method (DAKO LSAB2 kit) and substrate-chromogen reagent (3-amino-9-ethylcarbazole/H_2O_2, DAKO AEC Substrate System kit). Hepatic stellate cells in first and second passage were used for the present studies.

2.3. DNA synthesis ([^{3}H]thymidine incorporation)

Hepatic stellate cells in first passage were plated at a density of 1×10^4 cells/well in 24-well tissue culture plates and allowed to grow to be confluent in DMEM/F12 containing 20% fetal calf serum under standard tissue culture conditions. The confluent cells were growth-arrested in thymidine-free medium (DMEM containing 0.5% bovine serum albumin) for 48 h. Subsequently, cells were incubated with 25 ng/ml PDGF-BB (with 0.5% bovine serum albumin) and either vehicle or a given concentration of baicalein (3, 10, 30 or 100 μM), retinol (0.01, 0.1, 1 or 5 μM) or IBMX (50, 100, 300 or 500 μM). Cells were also incubated with 10% fetal calf serum and either vehicle or baicalein (3, 10, 30 or 100 μM). Either 12 h (with PDGF-BB stimulation) or 20 h (with fetal calf serum stimulation) later, cells were pulsed with [methyl-^{3}H]thymidine (1 μCi/ml), and at 24 h cells were washed with PBS and treated with 20% trichloroacetic acid at 4°C for 1 h. Precipitates were dissolved in 0.3 N NaOH/0.1% sodium dodecyl sulfate solution and radioactivity was determined by liquid scintillation counting. The viability of cells with the same treatments was assessed by a trypan blue-exclusion test.

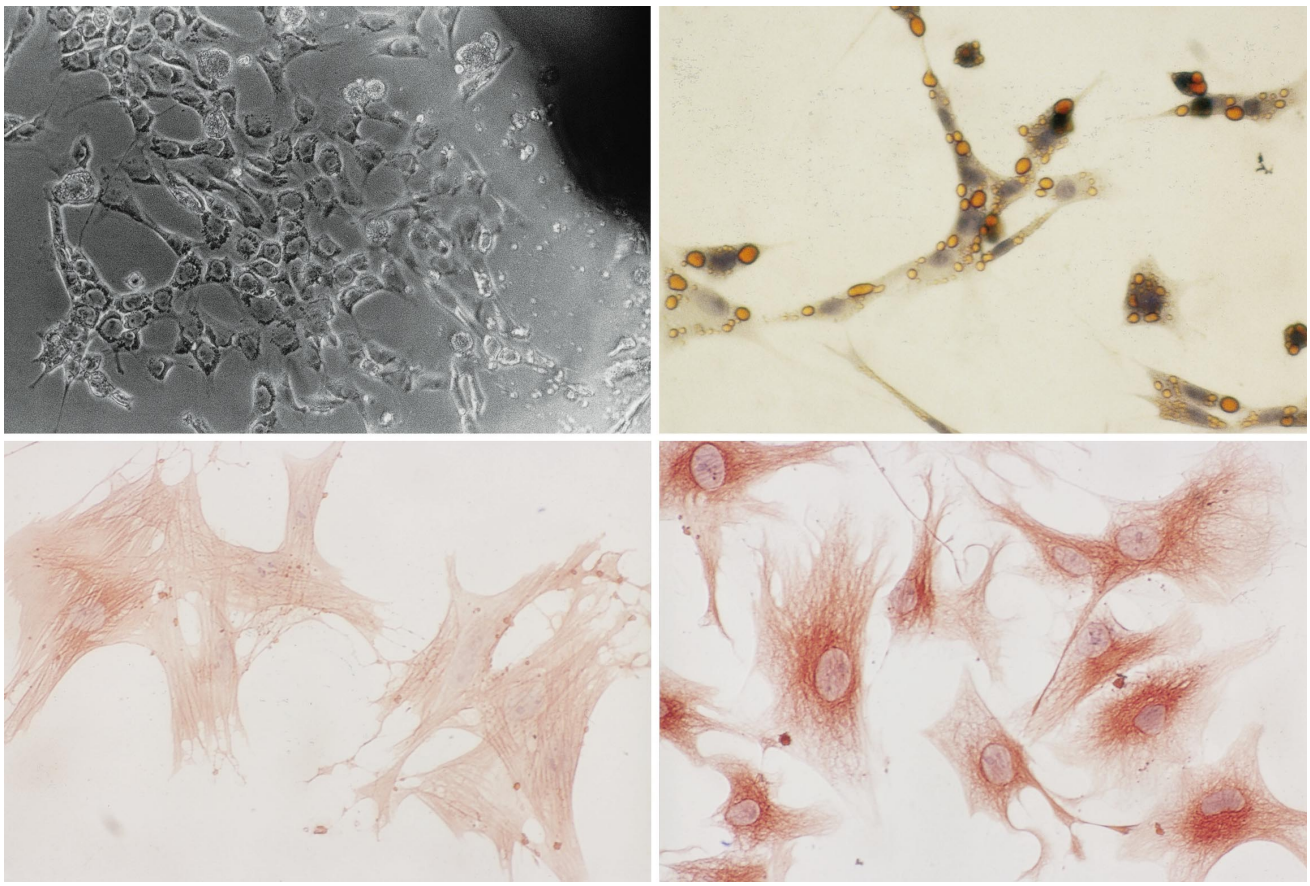

Fig. 1. Rat hepatic stellate cells obtained by explant culture. Top left, micrograph reveals a outgrowth-colony of hepatic stellate cells from liver tissue (right upper side) observed by light microscopy (original magnification; ×200) on the fifth day in primary culture. Cells abound with multiple cytoplasmic droplets which surround nuclei. Top right, phase-contrast micrograph of the cells (10th day in primary culture) stained with sudan IV reveals the lipid nature of the cytoplasmic droplets (orange) (original magnification; ×400). Cells were counterstained by Mayer's hematoxylin solution. Phase-contrast micrographs of the cells in first passage stained with mouse monoclonal antibodies to smooth muscle α-actin (bottom left) and vimentin (bottom right) reveal the presence of smooth muscle α-actin and vimentin filaments (original magnification; ×400). Cells were counterstained by Mayer's hematoxylin solution.

2.4. Cell number

Hepatic stellate cells in first or second passage were plated in 12-well plates (5×10^3 cells per well) and incubated in DMEM/F12 containing 20% fetal calf serum for 24 h. After 24 h (day 0), cells were treated with 1 ml DMEM/F12 containing 20% fetal calf serum and either vehicle or baicalein (3, 10 or 30 μM). Treatments were repeated every 2 days. At various times, cells were dislodged with 0.25% trypsin–EDTA and counted using a Coulter counter.

2.5. Protein synthesis ([^{3}H]leucine and [^{3}H]proline incorporation)

Confluent hepatic stellate cells in first passage were obtained in 24-well tissue culture plates as described for the DNA synthesis study (see above). The cells were treated with either leucine-free culture medium (DMEM [without leucine] containing 0.5% bovine serum albumin) or proline-free culture medium (DMEM containing 0.5% bovine serum albumin) for 48 h. Subsequently, the non-stimulated cells were incubated for 24 h with either the leucine- or proline-free culture medium containing 2.5% fetal calf serum supplemented with either L-[4,5-^{3}H(*N*)]leucine (1 μCi/ml) or L-[2,3,4,5-^{3}H]proline (1 μCi/ml) in the absence (vehicle) or presence of baicalein (3, 10, 30 or 100 μM). After 24 h of incubation, cells were washed with PBS. Half of the cells in each treatment were dislodged with 0.25% trypsin–EDTA and counted with a Coulter counter. The remaining cells were treated as described for the DNA synthesis study (see above), and radioactivity was determined by liquid scintillation counting. The radioactivity was normalized to cell number.

2.6. Analysis of data

Data are summarized as mean ± S.E.M. In the incorporation studies of radiolabeled precursors, data were compared using a one-factor analysis of variance (ANOVA)

followed by a Fisher's Protected Least Significant Difference test (Fisher's PLSD). In the cell number study, data were compared using a two-factor ANOVA followed by a Fisher's PLSD. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Effect of baicalein on DNA synthesis ([^{3}H]thymidine incorporation) with PDGF-BB stimulation

Baicalein markedly suppressed [^{3}H]thymidine incorporation in a concentration-dependent manner in hepatic stellate cells stimulated with 25 ng/ml PDGF-BB ($P < 0.0001$ by a one-factor ANOVA) (Fig. 2A). Baicalein at 10 μM decreased [^{3}H]thymidine incorporation approximately 63% (mean ± S.E.M., 5015 ± 200 dpm/well with 10 μM baicalein vs. 13,540 ± 711 dpm/well with vehicle). Baicalein at 100 μM decreased [^{3}H]thymidine incorporation approximately 81% closely to the value in non-stimulated cells treated with 0.5% bovine serum albumin alone (2568 ± 96 dpm/well with 100 μM baicalein vs. 1852 ± 79 dpm/well with 0.5% bovine serum albumin alone). As shown in Fig. 2B and C, retinol and IBMX, well-known antiproliferative agents in hepatic stellate cells, also suppressed [^{3}H]thymidine incorporation in these cells at generally employed concentrations ($P < 0.0001$). The 63% decrease observed with 10 μM baicalein was greater than those observed with the highest concentration of retinol or IBMX (38% with 5 μM retinol and 55% with 500 μM IBMX, respectively). The viability was over 95% in cells with the same treatments.

3.2. Effects of baicalein on proliferation ([^{3}H]thymidine incorporation and cell number) with fetal calf serum stimulation

As shown in Fig. 3A, baicalein strongly suppressed [^{3}H]thymidine incorporation in a concentration-dependent manner in hepatic stellate cells stimulated with 10% fetal calf serum ($P < 0.0001$ by a one-factor ANOVA). Even 3 μM baicalein decreased [^{3}H]thymidine incorporation up to 70% (mean ± S.E.M., 4511 ± 155 dpm/well with 3 μM baicalein vs. 14,791 ± 401 dpm/well with vehicle). Baicalein at 100 μM decreased [^{3}H]thymidine incorporation approximately 94% (915 ± 185 dpm/well with 100 μM baicalein). Baicalein also markedly suppressed increases in cell number in cells stimulated with 20% fetal calf serum in a concentration-dependent manner in both first (Fig. 3B) and second (Fig. 3C) passage ($P < 0.0001$ by a two-factor ANOVA). From day 2, after the first treatments with baicalein, the increases in cell number were significantly decreased by every concentration of baicalein. For instance, 51,633 ± 1142 (mean ± S.E.M.) cells/well in cells treated with vehicle vs. 37,281 ± 1129 cells/well in cells treated with 3 μM baicalein in first passage, and 53,242 ± 1991 vs. 40,562 ± 1719 cells/well in cells with the same treatments in second passage ($P < 0.001$ and $P < 0.01$ by a Fisher's PLSD, respectively). On day 6, approximately 40, 71 and 82% decreases were observed in cells treated with 3, 10 and 30 μM baicalein,

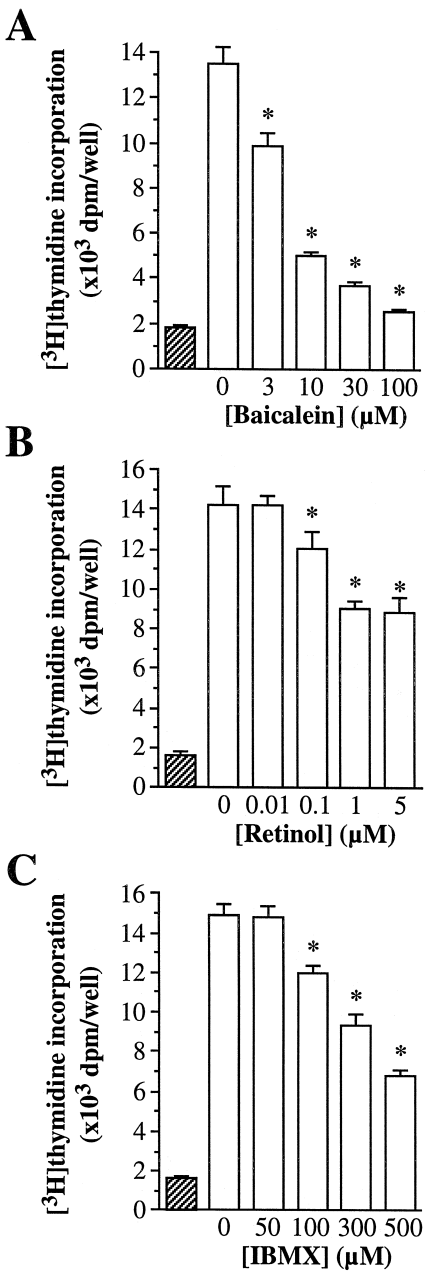

Fig. 2. Effects of baicalein, retinol and IBMX on DNA synthesis in hepatic stellate cells stimulated with PDGF-BB. [^{3}H]thymidine incorporation (an index of DNA synthesis) in cells (first passage) incubated for 24 h with either 0.5% bovine serum albumin alone (solid bar), or 25 ng/ml PDGF-BB with a given concentration of baicalein (A), retinol (B) and IBMX (C) (open bars). Asterisks indicate $P < 0.05$ compared with cells treated with vehicle (25 ng/ml PDGF-BB alone) by a one-factor ANOVA followed by a Fisher's PLSD. Values are means ± S.E.M. for eight observations.

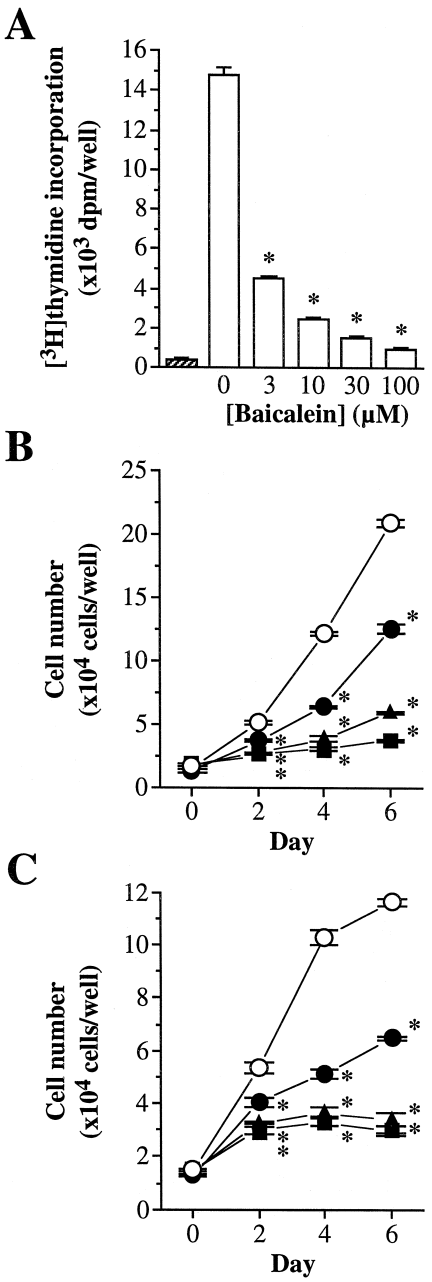

Fig. 3. Effects of baicalein on proliferation in hepatic stellate cells stimulated with fetal calf serum. (A) [^{3}H]thymidine incorporation in cells (first passage) incubated for 24 h with either 0.5% bovine serum albumin alone (solid bar), or 10% fetal calf serum with a given concentration of baicalein (open bars). Asterisks indicate $P < 0.05$ compared with cells treated with vehicle (10% fetal calf serum alone) by a one-factor ANOVA followed by a Fisher's PLSD. Values are means ± S.E.M. for eight observations. Line graphs depict increases in cell number, in first (B) and second (C) passage, when cells were incubated for 0 to 6 days with 20% fetal calf serum in the absence (vehicle, open circle) and presence of 3 (solid circle), 10 (solid triangle) or 30 μM baicalein (solid square). Asterisks indicate $P < 0.05$ compared with cells treated with vehicle (20% fetal calf serum alone) by a two-factor ANOVA followed by a Fisher's PLSD. Values are means ± S.E.M. for three observations in each passage.

respectively, in first passage (208,293 ± 3032 cells/well with vehicle vs. 125,353 ± 4148, 59,971 ± 431 and 37,407 ± 353 cells/well with 3, 10 and 30 μM baicalein, respectively, $P < 0.0001$). Hepatic stellate cells in second passage had a highly activated myofibroblast-like morphology during subculture with 20% fetal calf serum, i.e., large, hypertrophic and widely spread shapes with processes, losing cytoplasmic lipid droplets. However, similarly to the observations in first passage, approximately 45, 72 and 74% decreases were observed on day 6 in cells treated with 3, 10 and 30 μM baicalein, respectively (116,107 ± 1607 cells/well with vehicle vs. 64,547 ± 784, 32,353 ± 4344 and 29,967 ± 1676 cells/well with 3, 10 and 30 μM baicalein, respectively, $P < 0.0001$).

3.3. Effects of baicalein on protein synthesis ([^{3}H]leucine and [^{3}H]proline incorporation)

Baicalein moderately suppressed both [^{3}H]leucine (Fig. 4A) and [^{3}H]proline (Fig. 4B) incorporation in hepatic

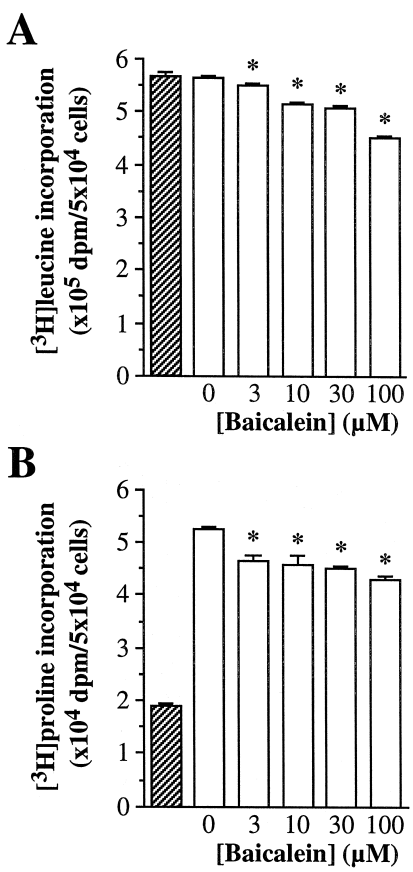

Fig. 4. Effects of baicalein on protein synthesis in hepatic stellate cells. [^{3}H]leucine incorporation (a general index of protein synthesis, A) and [^{3}H]proline incorporation (a general index of protein and collagen synthesis, B) in cells (first passage) incubated for 24 h with either 0.5% bovine serum albumin alone (solid bar), or 2.5% fetal calf serum with a given concentration of baicalein (open bars). Asterisks indicate $P < 0.05$ compared with vehicle (2.5% fetal calf serum alone) by a one-factor ANOVA followed by a Fisher's PLSD. Values are means ± S.E.M. for eight observations.

stellate cells stimulated with 2.5% fetal calf serum ($P < 0.0001$ by a one-factor ANOVA). Fetal calf serum at 2.5% did not induce a significant change in [^{3}H]leucine incorporation (a general index of protein synthesis) in these cells (mean ± S.E.M., 568,651 ± 6737 dpm/5×10^4 cells with 0.5% bovine serum albumin vs. 564,968 ± 3817 dpm/5×10^4 cells with 2.5% fetal calf serum). In contrast, 2.5% fetal calf serum increased [^{3}H]proline incorporation (a general index of protein and collagen synthesis) approximately threefold in these cells (19,084 ± 369 dpm/5×10^4 cells with 0.5% bovine serum albumin vs. 52,460 ± 441 dpm/5×10^4 cells with 2.5% fetal calf serum, $P < 0.0001$ by a Student's t test). The highest concentration of baicalein decreased [^{3}H]leucine and [^{3}H]proline incorporation approximately 20 and 18%, respectively (449,760 ± 3386 and 42,887 ± 786 dpm/5×10^4 cells with 100 μM baicalein, respectively, $P < 0.0001$ by a Fisher's PLSD). In these experiments, baicalein slightly reduced cell numbers during the 24 h incubation, up to 7.5 and 2.2% with 100 μM baicalein compared with vehicle in the [^{3}H]leucine and [^{3}H]proline incorporation study, respectively. The radioactivity was normalized to cell number in each study.

4. Discussion

The results in our DNA synthesis and cell number study, which were consistently observed in early passage, clearly demonstrate that baicalein has a strong antiproliferative effect in hepatic stellate cells. Moreover, baicalein suppressed moderately but significantly protein synthesis in hepatic stellate cells. These results indicate that the strong antiproliferative effect of this flavonoid, with an efficient inhibition of protein synthesis, may be importantly involved in the pharmacological mechanisms of the antifibrogenetic effect of Sho-saiko-to reported in experimental hepatic fibrosis (Amagaya et al., 1989; Miyamura et al., 1998; Sakaida et al., 1998; Shimizu et al., 1999).

In the DNA synthesis study with PDGF-BB stimulation, baicalein markedly suppressed [^{3}H]thymidine incorporation in a concentration-dependent manner in hepatic stellate cells. Retinoids (Davis and Vucic, 1988; Davis et al., 1990; Pinzani et al., 1992) and methylxanthine derivatives (Windmeier and Gressner, 1996; Kawada et al., 1996; Lee et al., 1997) are well-known strong antiproliferative agents in hepatic stellate cells. In our study, the decrease observed with 10 μM baicalein was greater than those observed with 5 μM retinol or 500 μM IBMX. This strong suppressive effect of baicalein on DNA synthesis was confirmed by the closely correlated observations in the [^{3}H]thymidine incorporation and cell number study with fetal calf serum stimulation. In a previous study with quercetin, a natural flavonoid chemically designated as 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one, which has the same basic chemical structure as baicalein with very minor differences in the positions of hydroxyls, quercetin inhibited proliferation and the expression of smooth muscle α-actin in cultured rat hepatic stellate cells, and suppressed the signal transduction pathway activated by PDGF-BB (Kawada et al., 1998). Moreover, quercetin arrested hepatic stellate cells at G1 phase with a selective decrease in the cellular level of cyclin D1, a cell cycle-related protein of G1 phase. Similar G1 arrest was observed with Sho-saiko-to in rat hepatic stellate cells (Kayano et al., 1998). In as much as quercetin and Sho-saiko-to activate the same antiproliferative mechanisms, i.e., G1 arrest in hepatic stellate cells, and since quercetin and baicalein have very similar chemical structures that both inhibit proliferation in hepatic stellate cells stimulated with PDGF-BB, baicalein may be an important ingredient for the antiproliferative effect of Sho-saiko-to in hepatic stellate cells.

The proliferation of activated hepatic stellate cells and the increased synthesis of extracellular matrix proteins by the cells are essential steps in the initiation and progression of hepatic fibrosis in chronic liver diseases (Friedman, 1993; Hautekeete and Geerts, 1997). PDGF is recognized to induce the activation and proliferation of hepatic stellate cells in injured livers (Pinzani et al., 1989; Win et al., 1993; Wong et al., 1994; Pinzani et al., 1996). Therefore, the inhibition of the activation and proliferation of hepatic stellate cells by Sho-saiko-to has been suggested as an important mechanism of the antifibrogenetic effect of this herbal preparation in experimental hepatic fibrosis (Miyamura et al., 1998; Sakaida et al., 1998; Shimizu et al., 1999). Recently, several studies suggested the involvement of oxidative stress in fibrogenesis of chronic liver disease (Tsukamoto et al., 1995; Pietrangelo, 1996; Baroni et al., 1998), and a very recent report indicated that Scutellariae radix-derived flavonoids in Sho-saiko-to may inhibit fibrogenesis in experimental hepatic fibrosis by their antioxidative activities (Shimizu et al., 1999). Presumably, both the antioxidative and antiproliferative activities of the flavonoids are important for the antifibrogenetic effect of Sho-saiko-to in hepatic fibrosis. In our studies, baicalein also suppressed protein synthesis in hepatic stellate cells. Even though the effect is moderate, this suppressive effect of baicalein on protein synthesis may also contribute to the antifibrogenetic effect of Sho-saiko-to in hepatic fibrosis.

In summary, we report the strong antiproliferative effects, with a moderate suppression of protein synthesis, of baicalein, a flavonoid derived from Scutellariae radix in Sho-saiko-to, in cultured rat hepatic stellate cells. These results indicate that baicalein is a very important ingredient for the antifibrogenetic effect of Sho-saiko-to observed in experimental hepatic fibrosis. Since Scutellariae radix has a long history as a common herbal medicine in China and Japan, flavonoids derived from Scutellariae radix may be safe and useful for the prevention and treatment of hepatic fibrosis in patients with chronic liver diseases.

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