

Conophylline Suppresses Pancreatic Stellate Cells and Improves Islet Fibrosis in Goto-Kakizaki Rats

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Activin A is a differentiation factor for β -cells and is effective to promote β -cell neogenesis. Activin A is also an autocrine activator of pancreatic stellate cells, which play a critical role in fibrogenesis of the pancreas. Conophylline (CnP) is a natural compound, which reproduces the effect of activin on β -cell differentiation and promotes β -cell neogenesis when administered *in vivo*. However, its effect on stellate cells is not known. We therefore investigated the effect of CnP on stellate cells both *in vitro* and *in vivo*. Unlike activin A, CnP inhibited activation of cultured stellate cells and reduced the production of collagen. We then analyzed the involvement of stellate cells in islet fibrosis in Goto-Kakizaki (GK) rats, a model of type 2 diabetes mellitus. In pancreatic sections obtained from 6-wk-old GK rats, CD68-positive macrophages and glial fibrillary acidic protein- and α -smooth muscle actin-positive stellate cells infiltrated into islets. Later, the number of macrophages was increased, and the α -smooth muscle actin staining of stellate cells became stronger, indicating the involvement of stellate cells in islet fibrosis in GK rats. When CnP was administered orally for 4 wk, starting from 6 wk of age, invasion of stellate cells and macrophages was markedly reduced and islet fibrosis was significantly improved. The insulin content was twice as high in CnP-treated rats. These results indicate that CnP exerts antifibrotic actions both *in vitro* and *in vivo* and improves islet fibrosis in Goto-Kakizaki rats. (*Endocrinology* 153: 621–630, 2012)

Islet fibrosis can be observed in the pancreas of patients with advanced diabetes. It can also be observed in various rodent models of type 2 diabetes mellitus (T2DM) (1–8). Goto-Kakizaki (GK) rats are nonobese, noninsulin-dependent diabetic rats characterized by hyposecretion of insulin (1). Kakizaki *et al.* (2) and others (3–5) described the presence of large disrupted islets separated by connective tissue in GK rats. In humans, pancreatic fibrosis was frequently found in diabetic patients with islet amyloid (9, 10). Fibrosis often follows inflammatory reactions, and the number of macrophages into islets was increased in GK rats and in patients with T2DM (5, 11).

Pancreatic stellate cells (PSC), which resemble hepatic stellate cells (12), is critical in fibrogenesis of chronic pancreatitis and pancreatic cancer. Fat-storing cells in the pancreas were first observed in 1982 (13). Two reports described identification, isolation, and characterization of PSC (14, 15). Since then, involvement of PSC in pancreatic fibrosis has been extensively studied. Quiescent PSC store vitamin A-containing lipid droplets in their cytoplasm. PSC express glial fibrillary acidic protein (GFAP) and desmin, an intermediate filament protein. Upon exposure to pancreatic damages and inflammation, PSC are transformed into activated myofibroblast-like cells. Activated PSC lose lipid droplets and express α -smooth muscle actin (α SMA). They migrate to sites of tis-

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Abbreviations: CCP-II, Crude CnP preparation II; CnP, conophylline; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GK, Goto-Kakizaki; GTT, glucose tolerance test; HE, hematoxylin-eosin; H+L, heavy and light chains; MafA, musculoaponeurotic fibrosarcoma A; MAPKAPK, mitogen-activated protein kinase-activated kinase; MCP-1, macrophage chemoattractant protein-1; PSC, pancreatic stellate cell; α SMA, α -smooth muscle actin; T2DM, type 2 diabetes mellitus.

sue damage, proliferate, phagocytose, and synthesize large amounts of extracellular matrix proteins leading to promotion of fibrosis (for review, see ref. 16).

Activin A acts as an autocrine activator of PSC and increases the expression of α SMA and collagen (17). In this regard, activin A may promote pancreatic fibrosis by activating PSC. On the other hand, activin A is a factor to promote differentiation of pancreatic β -cells. Thus, it induces differentiation of pancreatic AR42J cells and converts them to insulin-secreting cells (18). Activin A also promotes regeneration of pancreatic β -cells and improves glucose metabolism in neonatal streptozotocin-treated rats (19, 20). Despite its efficacy *in vivo*, activin A has adverse effects, for example, induction of apoptosis in some types of cells (21). We attempted to search for a new differentiation-inducing compound that mimics the action of activin A and found the compound conophylline (CnP) (22). CnP is a vinca alkaloid extracted from leaves of a tropical plant (23). It promotes differentiation of β -cells and improves glucose metabolism in animal models of diabetes (24, 25). Unlike activin A, CnP does not induce apoptosis (22). Hence, it may be a potential candidate factor in promotion of β -cell regeneration *in vivo*. In this regard, determination of whether or not CnP activates PSC as activin A does and whether it promotes pancreatic fibrosis is a critical issue.

In the present study, we investigated the effect of CnP on activation of PSC. We also examined the effect of CnP *in vivo* using GK rats. Our results clearly show that, unlike activin A, CnP inhibits activation of PSC and improves islet fibrosis.

Materials and Methods

The CnP was purified from leaves of *Ervatamia microphylla* (23). The Crude CnP preparation II (CCP-II) used in the *in vivo* study was obtained from leaves of *Tabernaemontana divaricata* (25). Briefly, dry leaves (5 kg) were added to 500 liters of 0.025 N hydrochloric acid and heated to 60 C for 30 min. Then, a clear supernatant was obtained by centrifugation. The supernatant was subjected to column chromatography using a synthetic absorbent resin, Diaion HP20 (Mitsubishi Chemical, Tokyo, Japan). The column was washed with water followed by 30% ethanol, and CnP was then eluted with pure ethanol. The elute was concentrated *in vacuo* and lyophilized. Major contaminant in CCP-II is conophyllide (25), a related compound with similar activity as CnP. Consequently, CCP-II is as potent as purified CnP or slightly more potent compared with CnP when equivalent amount is administered. CCP-II contained 22 mg/g of CnP. Recombinant human activin A was kindly provided by Y. Eto (Ajinomoto Co., Inc., Kawasaki, Japan). Recombinant human TGF β ₁ was purchased from R&D Systems, Inc. (Minneapolis, MN). Pronase and Nycodenz were from Sigma-Aldrich Co. (St. Louis, MO).

Isolation and culture of rat pancreatic stellate cells

All animal experiments were performed under the permission of the Animal Care and Experimentation Committee, Gunma University.

Wistar rats were purchased from Clea Japan, Inc. (Tokyo, Japan). PSC were isolated from adult male Wistar rats by the method described by Apte *et al.* (14). PSC were resuspended in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum (FBS), 4 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. PSC were cultured in a 5% CO₂ atmosphere at 37 C. All the experiments were performed using cells between passages two and five.

Western blotting

After growing to confluence, PSC were subcultured at equal densities and incubated with Iscove's modified Dulbecco's medium containing 10% FBS for half a day. After serum deprivation for 24 h, some agents were added to the medium. For *in vitro* study, pure CnP was used. CnP was dissolved in MeOH, and the final concentration of MeOH in culture medium was 0.1%. After 48 h of incubation, cultured PSC were scraped off and collected using 2 \times sodium dodecyl sulfate sample buffer [125 mM Tris-HCl (pH 6.8), 4% wt/vol sodium dodecyl sulfate, and 20% glycerol]. Dithiothreitol at a final concentration of 50 mM was added after the protein assay. Protein concentrations were measured by a commercial kit (BCA Protein Assay kit; Pierce, Rockford, IL) using BSA as standard. Protein samples (2 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride microporous membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was blocked with 5% skim milk in PBS containing 0.05% Triton X-100 for 1 h at room temperature and was then incubated overnight at 4 C with the primary antibodies, anti- α SMA antibody (Sigma-Aldrich Co.) diluted 1:10,000 and anti- β -tubulin antibody (MP Biomedicals, LLC, Morgan Irvine, CA) diluted 1:2000. After the membranes were washed with PBS containing 0.05% Triton X-100, they were incubated with secondary antibody (1:2000) for 1 h at room temperature. Antibody reactive was detected using an enhanced chemiluminescence (ECL) system (ECL for α SMA, ECL-plus for β -tubulin; GE Healthcare, Buckinghamshire, UK), and images were obtained by a multipurpose charge-coupled device camera system (LAS 4000; Fujifilm Co., Tokyo, Japan).

Immunocytochemistry

PSC were cultured on noncoated glass coverslips at a density of 2×10^4 /ml. Cells were fixed with 10% neutral buffered formalin, treated with 0.1% (vol/vol) Triton X-100 in PBS for 10 min, and incubated for 1 h with Block Ace (DS Pharma Biomedical Co, Osaka, Japan). After washing, they were incubated with Alexa Fluor 488 goat antimouse IgG [heavy and light chains (H+L)] conjugate (1:200) (Invitrogen, Carlsbad, CA) for 1 h and then with 4' diamino-2-phenylindole (Pierce).

Measurement of DNA synthesis

DNA synthesis was assessed by measuring [³H]thymidine incorporation. After a 24-h serum deprivation, PSC were cultured in various conditions for 24 h, and 0.5 μ Ci/ml [³H]thymidine was included in the last 4 h. Cells were washed twice with ice-cold PBS and solubilized, and radioactivity associated with trichloroacetic acid-precipitable materials was counted (17).

Assay of collagen secretion

Collagen secreted into culture medium was determined by a dye-binding method using Sircol collagen assay (Biocolor Ltd, Carrickfergus, UK). After a 24-h serum deprivation, PSC were incubated in the presence of some reagents in a medium containing 5% FBS for 48 h. Then, 100- μ l samples were added to 1 ml of Sircol dye, followed by centrifugation to pack the collagen-dye complex at the bottom of the tube. The pellet obtained was dissolved in an 1-ml alkali reagent. Collagen was measured by spectrophotometry at 550 nm.

Measurement of macrophage chemoattractant protein-1 (MCP-1)

PSC were cultured in the same condition for assay of collagen secretion. MCP-1 in the culture medium was measured by ELISA (Endogen).

Measurement of p38 activation

The levels of activated, phosphorylated p38 MAPK were determined by Western blotting using the phosphoPlus p38 MAPK (Thr180/Try182) Antibody kit (Cell Signaling, Danvers, MA) according to the manufacturer's instructions. After 24 h of deprivation of serum, PSC were cultured with 100 ng/ml CnP for various periods. The effect of specific inhibitor of p38, SB203580, on the phosphorylation of mitogen-activated protein kinase-activated kinase (MAPKAPK-2), a downstream target of p38, was examined. Serum-deprived PSC were treated with SB203580 for 30 min and then stimulated with 10% serum for 60 min. Antiphospho-MAPKAPK-2 (Thr222) antibody was from Cell Signaling.

Animals

Male GK rats and Wistar rats were purchased from Nihon SLC (Hamamatsu, Japan). They were kept in an air-conditioned environment, with a 12-h light, 12-h dark cycle, and fed *ad libitum* a commercial standard pellet diet (Clea Japan, Inc.). For investigation of the involvement of PSC in islet fibrosis, pancreases of various ages were removed and fixed at 4 C in 10% neutral buffered formalin for 24 h and embedded in Paraplast Plus (McCormick Scientific LLC, St. Louis, MO).

Treatment with CnP (CCP-II)

Male GK rats 6 wk of age were divided into two groups, and each group consists of five rats. The CnP group was treated with 0.9 μ g/g of CnP and the control group with vehicle via an oral cannula every day for 4 wk. In the *in vivo* study, CCP-II were used. CCP-II was suspended uniformly in water containing 0.5% Tween-80 solution.

Nonfasting blood glucose concentrations obtained every week in samples from the tail vessels were measured by a glucose oxidase method (FreeStyle, Nipro, Osaka, Japan). An ip glucose tolerance test (GTT) was performed before and after the treatment. At the end, blood was withdrawn from the heart under anesthesia, and the whole pancreas was removed and weighed. A part of the pancreas (upper-tail area) was used for measurement of the insulin content, and the rest of the pancreas was stored overnight in 4 C 10% neutral buffered formalin for 24 h before routine paraffin wax processing.

Intraperitoneal GTT

An ipGTT was performed after an overnight fast; 2 g/kg of glucose were injected into the peritoneum after blood sampling. Blood samples were collected from the tail at 30, 60, and 120 min after the injection.

Measurement of insulin content and serum insulin level

A part of the pancreas was weighed and then homogenized in cold acid-ethanol, shaken overnight at 4 C, heated for 5 min in a 70 C water bath, and centrifuged, and the supernatant was then stored at –20 C until assay. The insulin content and serum insulin level were measured by RIA using an insulin RIA kit (Eiken, Tokyo, Japan). The insulin content was normalized by the weight of the pancreas.

Histological examination

Each paraffin-embedded block was serially sectioned (5 μ m). Serial sections were stained with hematoxylin-eosin (HE), Masson's trichrome, antibodies against insulin, glucagon, CD68, α SMA, GFAP, and masculoaponeurotic fibrosarcoma A (MafA). Sections for immunostaining were incubated with 0.3% H₂O₂ in MeOH for 30 min to block endogenous peroxidases and 1% BSA in PBS for 30 min to prevent nonspecific binding of antibody. Primary antibodies were used at the following dilutions: guinea pig anti-swine insulin, 1:1000 (Dako, Glostrup, Denmark); rabbit anti-human glucagon, 1:1000 (Dako); mouse anti-rat CD68 (ED1), 1:200 (AbD Serotec, Oxford, UK); mouse anti-human SMA, 1:100 (Dako); rabbit anti-GFAP, 1:500 (Dako); and rabbit anti-MafA 1:100 (Bethyl, Montgomery, TX). The following immunostaining systems were used: biotinylated anti-guinea pig IgG (H+L), 1:200 (Vector Laboratories, Inc., Burlingame, CA); horseradish peroxidase streptavidin, 1:200 (Vector Laboratories, Inc.); Histofine Simple Stain Rat MAX PO (MULTI) (Nichirei Co., Tokyo, Japan); alkaline phosphatase conjugated donkey anti-rabbit IgG (H+L), 1:200 (Chemicon International, Inc., Temecula, CA); DakoCytomation Liquid DAB Substrate Chromogen System (Dako); and Vector Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories, Inc.). Sections were counterstained with hematoxylin. To count MafA-positive cells, slices were stained with anti-MafA and anti-insulin antibodies, and the numbers of insulin-positive cells and MafA/insulin double-positive cells were counted. At least 10 islets were counted for each section, and five sections were selected per animal.

Quantitative analysis of fibrosis in islets

To calculate the relative percentage of blue-stained area within islets in Masson's trichrome-stained sections and areas at the same time, each section was processed using ImageJ 1.43 image analysis software (National Institute of Health, Bethesda, MD) by means of a CX41 microscope (Olympus, Tokyo, Japan) with a DP21 digital camera (Olympus). The amount of collagen deposition was calculated from 100 nonoverlapping islets per pancreas. The amount of islet fibrosis is presented as a percentage of the total islet area and determined as the (area of blue stained/total area of islet) \times 100.

Measurement of β -cell mass

To determine the β -cell mass, the area of insulin-positive cells was measured using ImageJ. Nonoverlapping but almost whole sections (at least 20 fields per section; magnification, $\times 40$, three sections per rat) were measured. To calculate the β -cell mass, this relative volume of β -cell was multiplied by the pancreatic weight.

Statistical analysis

Results were expressed as means \pm SE. For comparison between the two groups, an unpaired *t* test was used. $P < 0.05$ was considered to be significant. To analyze data obtained by GTT, ANOVA was used.

Results

Effect of CnP on cultured PSC

We examined whether or not CnP affected the activation of PSC. We first examined the effect of CnP by immunocytochemistry. As shown in Fig. 1A, the stress fiber-like structure was disrupted by CnP, and the expression of α SMA was reduced in a dose-dependent manner. We quantified the expression of α SMA by immunoblotting. As depicted, CnP suppressed the expression of α SMA, whereas TGF β_1 and activin A increased the expression of α SMA (Fig. 1B). We next examined the effect of CnP on DNA synthesis by measuring [3 H]thymidine incorporation. As shown in Fig. 1C, thymidine incorporation induced by 10% serum was markedly decreased by CnP. At a concentration of 100 ng/ml, CnP almost completely blocked DNA synthesis. Note that the viability of PSC was not affected at this concentration. TGF β_1 or activin A showed no effect (data not shown). We then measured secretion of collagen into culture medium. As shown in Fig. 1D, CnP considerably reduced the secretion of collagen. This effect of CnP was completely different from that by activin A, which augments collagen production (17). CnP also inhibited production of MCP-1, a chemokine which stimulates migration of macrophages (Fig. 1E).

The above results indicate that the effects of CnP on PSC is completely different from those of activin A. We previously showed that CnP reproduced the differentiation-inducing activity of activin by stimulating the expression of neurogenin-3. This effect of CnP and activin A is exerted through activation of p38 MAPK (22). We therefore examined the involvement of p38 in the action of CnP on PSC. As shown in Fig. 2A, CnP slowly but significantly activated p38. To block the activity of p38, we used SB203580. Figure 2B shows that SB203580 nearly completely inhibited p38-mediated phosphorylation of MAPKAPK-2, a substrate of p38, at a concentration of 5 μ M. Using this compound, we then assessed whether or not the effects of CnP on PSC were mediated by p38. As depicted

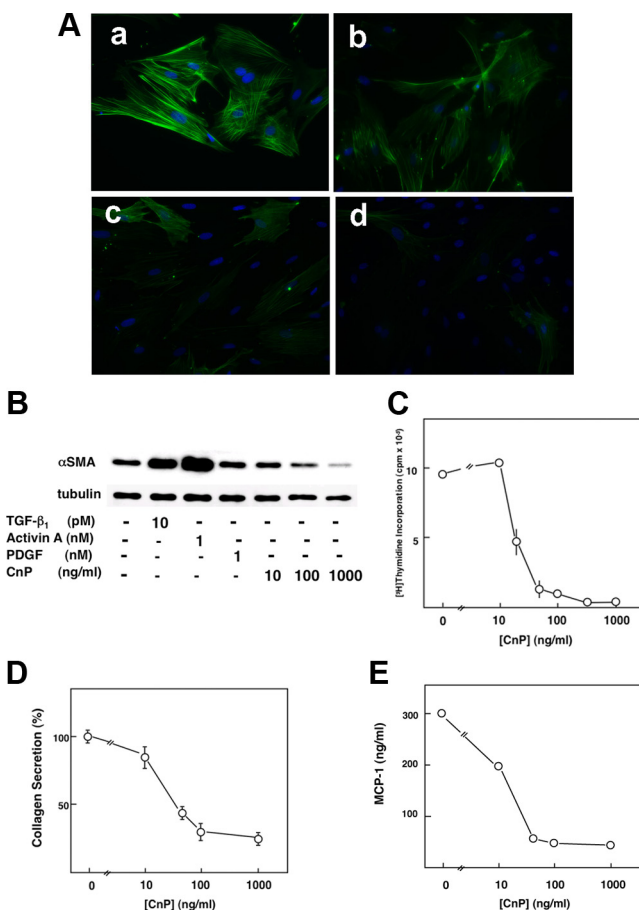


FIG. 1. Effect of CnP on the expression of α SMA, DNA synthesis, and secretion of collagen and MCP-1 in cultured PSC. **A**, After 24 h of serum starvation, 0 (a), 0.01 (b), 0.1 (c), and 1 μ g/ml (d) CnP were added into the culture medium. PSC were cultured for 48 h and fixed. PSC were stained with anti- α SMA (green) and 4' diamino-2-phenylindole (blue). All immunofluorescence images were taken at the same magnification ($\times 200$). **B**, PSC were incubated for 48 h with various agents, and the expression of α SMA was measured by immunoblotting. Expression of α SMA and anti- β -tubulin as the control. **C**, DNA synthesis was assessed by measuring [3 H]thymidine incorporation. After 24 h of incubation in serum-free medium, PSC were cultured with various agents in the presence of 5% serum, and [3 H]thymidine incorporation was measured. Values are the mean of four experiments. **D**, Effect of CnP on collagen secretion from PSC. PSC were cultured for 48 h with various concentrations of CnP, and collagen secreted to the medium was measured. Values are the mean \pm SE for four experiments. **E**, Effect of CnP on the Production of MCP-1. PSC were cultured for 48 h with various concentrations of CnP, and MCP-1 in the medium was measured. Values are the mean \pm SE for four experiments. PDGF, Latelet-derived growth factor.

in Fig. 2C, the inhibitory effect of CnP on DNA synthesis was not affected by SB203580. Likewise, SB203580 did not block the inhibitory effect of CnP on the expression of α SMA (Fig. 2D).

Involvement of PSC in islet fibrosis in GK rats

We next investigated the involvement of PSC in islet fibrosis in GK rats. We prepared pancreatic serial sections obtained from GK rats of various ages and stained with

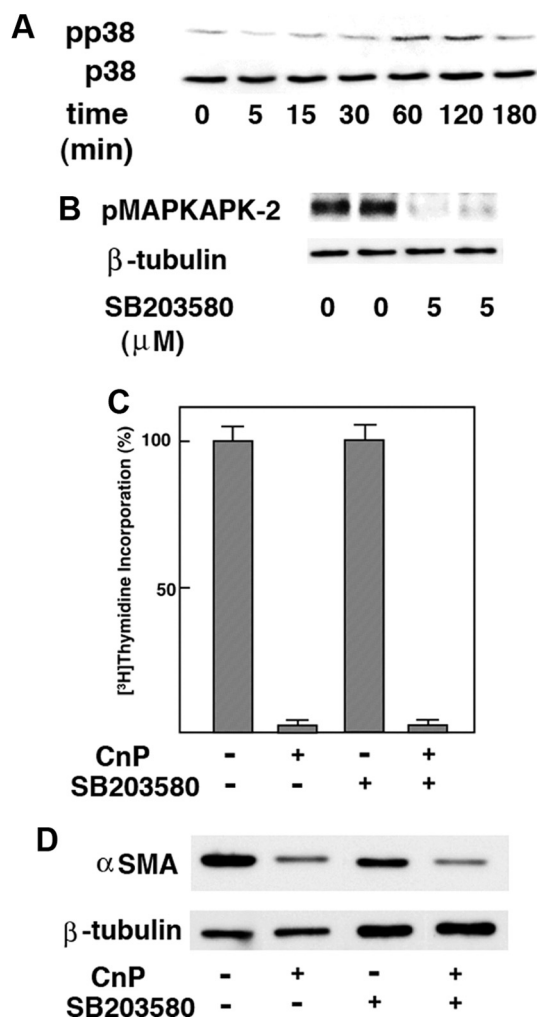


FIG. 2. Involvement of p38 in the action of CnP in PSC. A, PSC were incubated for various time periods with 100 ng/ml CnP, and changes in the expression of phosphorylated p38 (pp38) were measured by immunoblotting. B, PSC were incubated for 60 min with 10% serum in the presence and absence of 5 μM SB203580. The amount of pMAPKAPK-2 was measured by immunoblotting. C, PSC were incubated for 24 h with 10% serum-containing medium with or without 100 ng/ml CnP in the presence and absence of 10 μM SB203580 and [³H]thymidine incorporation was measured. Values are the mean ± se for four experiments. D, PSC were incubated for 48 h in serum-free medium with or without 100 ng/ml CnP in the presence and absence of 10 μM SB203580. The expression of αSMA was measured by immunoblotting.

HE or antibodies against insulin/glucagon, CD68, αSMA, and GFAP. CD68 and GFAP are markers for macrophages and PSC, respectively. αSMA is a marker for activated PSC. All islets were morphologically normal and were not stained with anti-CD68, αSMA, or GFAP before 5 wk of age (Fig. 3). At 6 wk, CD68-positive macrophages were observed mainly around islets, and αSMA and GFAP-positive PSC were observed in these islets, although the islets were morphologically unchanged. Islets 7 wk old had irregular contour. The core of islet was segmented into small clusters, each of which was surrounded by fibrous tissues.

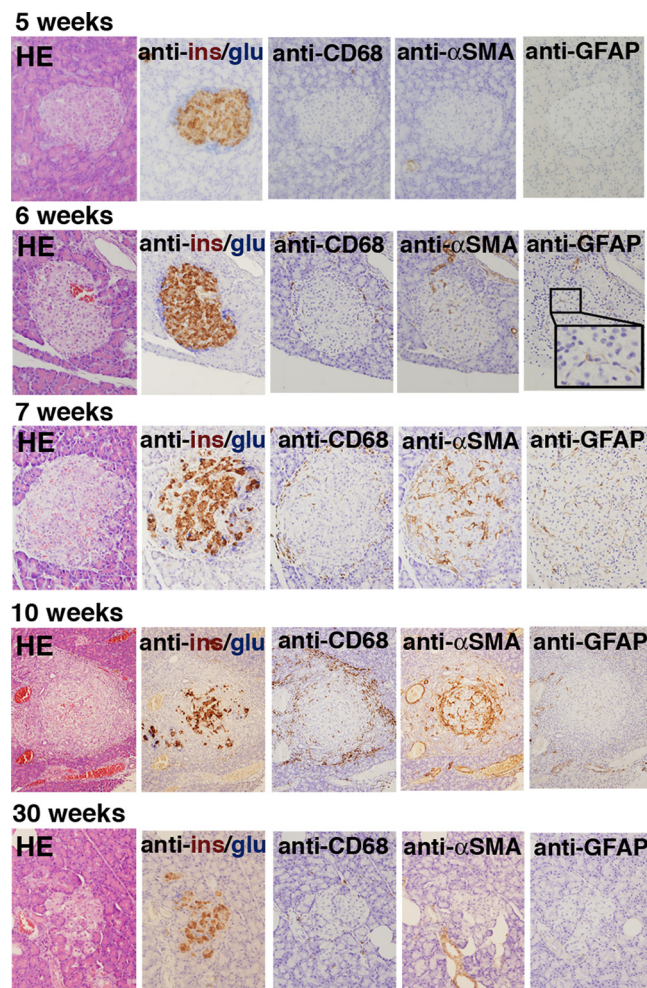


FIG. 3. Localization of PSC in Islet of GK rats. Pancreatic serial sections obtained from GK rats of 5, 6, 7, 10, and 30 wk old were stained with HE, anti-insulin (brown) and glucagon (blue) (anti-ins/glu), anti-CD68, anti-αSMA, and anti-GFAP. Nuclei were stained with hematoxylin.

The number of macrophages was increased, and most of them were in the periphery of the islets. The number of PSC was increased markedly. Thereafter, the islets were disrupted and surrounded by numerous macrophages. αSMA staining became much stronger, whereas immunoreactivity of GFAP was rather decreased. Such inflammatory changes were restricted to inside and around the islets and other areas, including acinar cells, which were apparently normal. In a 30-wk-old rat, infiltration of macrophages and PSC into or around islets disappeared.

To examine the presence of PSC in detail, we compared islets of 6-wk-old GK and Wistar rats when PSC appear in islets. Each pancreatic serial section was stained with anti-GFAP. There were no GFAP-positive cells in the islets of Wistar rats (Fig. 4A). In contrast, each islet was surrounded by GFAP-positive cells in GK rats, and one GFAP-positive cell was just migrating into the islet (Fig. 4B, arrow).

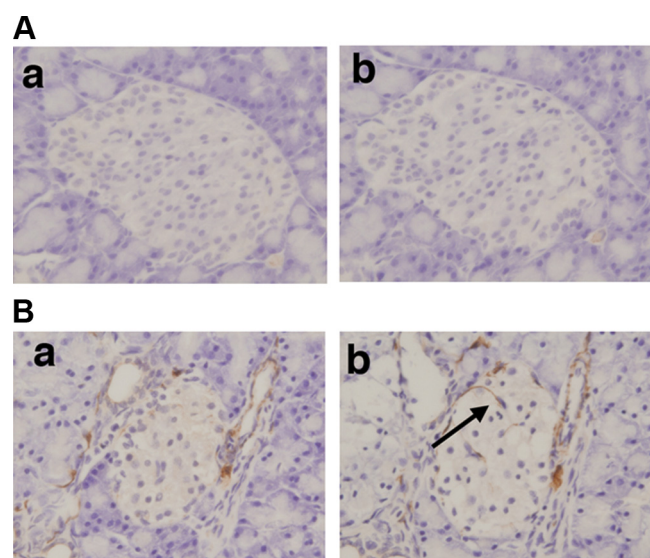


FIG. 4. Comparison of GFAP-positive cells in 6-wk-old GK and Wistar Rats. Islets of GK (B) and Wistar (A) rats were stained with anti-GFAP antibody. Two serial sections are shown in each rat (a and b). GFAP-positive cells were stained in brown ($\times 400$). The arrow shows a GFAP-positive cell just invading into an islet.

Effect of CnP on islet fibrosis and glucose metabolism in GK rats

We then examined whether or not administration of CnP inhibited islet fibrosis and affected glucose metabolism in GK rats. At 6 wk of age, PSC initiate invasion into islets. Therefore, we started administration of CnP at this time point. We orally administered $0.9 \mu\text{g/g}$ CnP or vehicle for 4 wk. For *in vivo* experiments, we used CCP-II (25). It should be mentioned that CCP-II also inhibited *in vitro* the activation of PSC as did CnP (data not shown).

There was no difference in body weight and the blood glucose concentrations in CnP-treated and control groups during the experiment. After 4 wk of treatment, the random plasma glucose concentration and the insulin concentration were not significantly different in CnP-treated rats (Table 1). We then performed a GTT at 10 wk. The plasma glucose concentration at 60 min after the ip injection of glucose was slightly decreased in CnP-treated rats (Fig. 5A), but the difference was not statistically significant. In these rats, the serum insulin concentrations tended to be high, and the insulin content of the pancreas was twice as high compared with that in control rats (Fig. 5B).

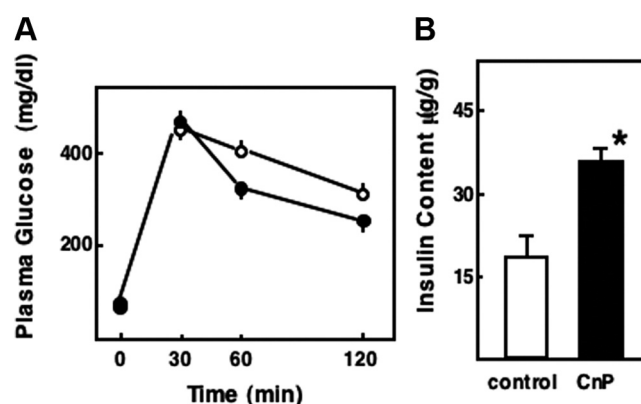


FIG. 5. Effect of Administration of CnP on glucose metabolism. A, An ipGTT was performed after 4 wk of treatment with CnP. Changes in the plasma glucose concentration in CnP-treated (●) and control (○) rats are presented. B, Insulin content was measured in the pancreas in GK rats after 4 wk of treatment. Values are the mean \pm SE for four experiments. *, $P < 0.05$ vs. control.

Histological analysis was performed using pancreatic sections obtained from 10-wk-old rats. The islets of control GK rats were enlarged, and proliferation of connective tissue and deposit of collagen fiber were observed (Fig. 6Aa). In contrast, the area of blue staining in Masson's trichrome-stained section, which represents the deposit of collagen fiber, was only observed in the perivascular area in CnP-treated rats (Fig. 6Ab).

The islets of control GK rats had irregular contour and were intensely stained in blue (Fig. 6B). β -Cells in the core of the islets were segmented into small clusters, which were surrounded by collagen fibers. α SMA was stained strongly in islets, and many CD68-positive cells were observed in and around the islets. The islets of CnP-treated rats were considerably different. The fibrosis of the islets was much improved, and fragmentation of β -cells in the core of islets was not observed. The numbers of α SMA and CD68-positive cells were smaller compared with those in control rats. Some islets were morphologically almost normal. The blue staining area was hardly observed in these islets. In addition, CD68-positive cells and α SMA-positive cells were rarely observed in and around the islets, and the mantle-core structure was maintained. The ratio of the fibrotic area to the total islet area was calculated. As shown in Fig. 7A, CnP treatment slightly reduced the islet

TABLE 1. Effect of CnP in GK rats

	Body weight (g)	Pancreas weight (g)	Plasma glucose (mg/dl)	Serum insulin (mU/ml)
Control	211 \pm 4.8	0.58 \pm 0.017	177 \pm 41.3	50.7 \pm 13.5
CnP	214 \pm 8.7	0.70 \pm 0.016 ^a	141 \pm 7.5	62.4 \pm 6.6

CnP ($0.9 \mu\text{g/g}$) or vehicle was administered orally to GK rats for 4 wk starting at the age of 6 wk. On wk 10, rats were killed, and the body weight, pancreas weight, plasma glucose concentration, and serum insulin concentration were measured. Values are the mean \pm SE for four experiments.

^a $P < 0.01$ vs. control.

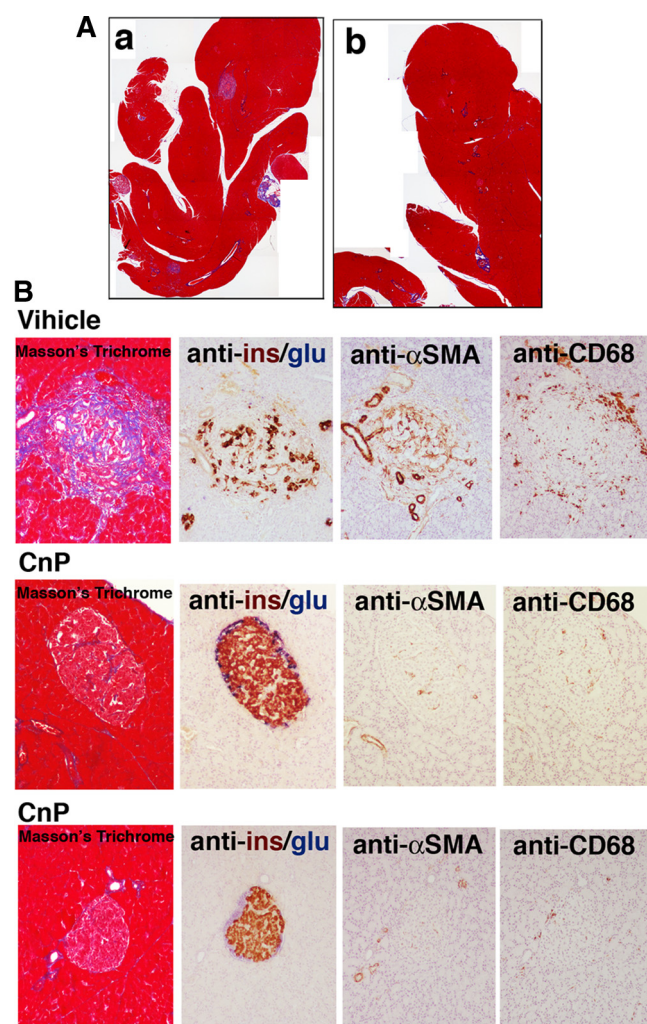


FIG. 6. Effect of CnP on the production of collagen. A, After 4 wk of treatment with CnP (b) or vehicle (a), pancreas sections were stained with Masson's trichrome. Low-magnification ($\times 40$) images are combined to represent whole pancreas. B, After 4 wk of treatment with CnP or vehicle, pancreas serial sections were stained with Masson's trichrome or antibodies against insulin (brown) and glucagon (blue) (anti-ins/glu), α SMA, and CD68. All images shown are in the same magnification ($\times 100$).

area, but the effect was not statistically significant. Treatment with CnP for 4 wk significantly reduced islet fibrosis (Fig. 7B). Although the insulin content was much higher in CnP-treated rats, the β -cell mass in these rats was not different from that of control rats (Fig. 7C). The number of Ki67-positive β -cells in islets was not different between the two groups (data not shown). We also measured the number of terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick end labeling-positive cells in the islets, and there was no difference in the number of terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick end labeling-positive cells between the two groups (data not shown). We also measured the number of MafA-positive β -cells in the islets. As shown in Fig. 7D, the expression of MafA in β -cells was increased

in CnP-treated GK rats, and the number of MafA-positive β -cells was greater in CnP-treated GK rats (Fig. 7E). Insulin-positive ductal cells were found in both control and CnP-treated rats. The number of insulin-positive ductal cells was not different between the two groups, and the number of islet-like cell clusters was nearly identical between the two groups (data not shown).

Discussion

Activated PSC express α SMA and type I collagen (26, 27) and play a critical role in pancreatic fibrosis associated with chronic pancreatitis (26–28). Islet fibrosis is also observed in various rodent models of T2DM, including GK rats. We immunohistochemically investigated the involvement of PSC in islet fibrosis in GK rats. Based on our observation, the changes in islets can be classified into the following stages. 1) Normal stage: all islets are morphologically normal and not stained with α SMA, GFAP, or CD68, despite the fact that glucose levels in ipGTT have already been impaired at 3 wk of age (data not shown). 2) Infiltration stage: islets are morphologically unchanged except that CD68-positive macrophages are observed around islets, and some α SMA and GFAP-positive PSC were observed in islets. 3) Inflammation stage: islets have irregular contour, the core of islet β -cells are segmented into small clusters, and the numbers of macrophages and PSC are increased. 4) Advanced inflammation stage: islets are disrupted remarkably and are surrounded by many macrophages; α SMA staining becomes stronger. 5) Final stage: islets become starfish-like; infiltration of macrophages and PSC disappeared.

We do not have a definite answer as to what initiates activation of PSC in GK rats, but previous studies have suggested that high concentrations of glucose, hydrogen peroxide, and nicotinamide adenine dinucleotide phosphate oxidase, which are sources of reactive oxygen species, activate PSC (29–33). Activated PSC produce MCP-1, and macrophages migrate around PSC (34, 35). Macrophage and macrophage-related inflammatory cells produce cytokines, which further activate PSC. It is strongly suggested that activated PSC invade into islets of GK rats and promote inflammation and production of matrix proteins (36). It should be mentioned that we identified stellate cells by detecting marker proteins such as α SMA and GFAP. In a strict sense, we cannot exclude the possibility that islet fibrosis is caused by similar type of cells expressing identical marker proteins, including circulating fibrocytes (37). Nevertheless, because we detected invasion of GFAP-positive cells into islets (Fig. 4B),

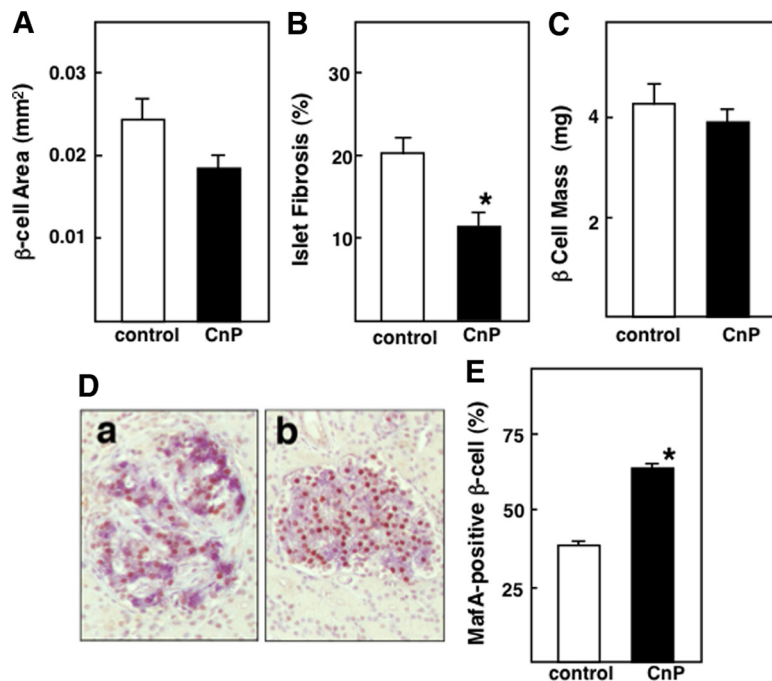


FIG. 7. Effect of CnP on islet fibrosis and the β -cell mass. A, The mean islet area was measured in CnP- and vehicle-treated GK rats. Values are the mean \pm SE ($n = 4$). B, Pancreatic sections were stained with Masson's trichrome, and the amount of islet fibrosis calculated as $100 \times (\text{area of blue staining})/(\text{total islet area})$ is presented. Values are the mean \pm SE for four determinations. *, $P < 0.05$ vs. control. C, The β -cell mass was calculated in specimen obtained from CnP- and vehicle-treated GK rats. D, Pancreatic sections obtained from CnP-treated (b) and vehicle-treated GK rats (a) were stained with anti-MafA (brown) and antiinsulin (purple) antibodies. E, The number of MafA-positive β -cells was counted in CnP- and vehicle-treated islets. Values are the mean \pm SE. *, $P < 0.001$ vs. control.

most of the GFAP-positive cells islets were PSC invaded into islets.

The most intriguing and unexpected results obtained in this study are the effects of CnP on PSC activation. Although CnP reproduces the effect of activin A on differentiation of pancreatic progenitor cells, CnP has an opposite effect on PSC activation. Thus, CnP inhibited DNA synthesis in PSC, reduced the expression of α SMA, and inhibited the production of collagen and MCP-1. The results obtained in the previous studies indicate that CnP reproduces the differentiation-inducing action of activin A by activating p38, which is critical in the induction of neurogenin-3 expression (22). Indeed, the effects of CnP on PSC were not affected by an inhibitor of p38. Thus, the actions of CnP on PSC were not mediated by p38. At present, we have not yet identified the mechanism by which CnP inhibits PSC activation. In this regard, Atsumi *et al.* (38) showed that CnP suppressed the TGF β signaling by up-regulation of c-Jun expression. Further studies are needed to identify the mechanism of action of CnP in PSC. In any event, CnP attenuates activation of PSC not only in an *in vitro* condition but also in an *in vivo* con-

dition. CnP significantly reduced islet fibrosis and increased the insulin content. It has been shown that peroxisome proliferator-activated receptor- γ (35), blockade of the rennin-angiotensin system (39), follistatin (40), serine-protease inhibitors, and an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase (33) inhibit PCS activation *in vitro*. Of these, peroxisome proliferator-activated receptor- γ ligands (41) and a blockade of rennin-angiotensin system (42, 43) suppress islet fibrosis in animal models of T2DM. CnP is another candidate compound that prevents islet fibrosis.

It should be mentioned that the dose of CnP used in this study is modest. We administered $0.9 \mu\text{g/g}$ CnP for 4 wk because of the limited amount of purified CnP available. At this CnP dose, we could not demonstrate that CnP promotes differentiation of pancreatic β -cells or stimulates neogenesis of β -cells. There were no differences in the numbers of PDX-1-positive cells in the duct, insulin-positive cells in the duct, and islet-like cell clusters in the control and CnP-treated rats. CnP did not significantly promote neogenesis of β -cells at least at the dose employed in this study. If we had administered a higher amount of CnP for a longer period, we could have observed a higher effect in GK rats. In this regard, the expression of MafA was up-regulated in β -cells of CnP-treated GK rats. This may explain at least partly the increase in the insulin content in CnP-treated GK rats.

In summary, involvement of PSC in islet fibrosis was strongly suggested in GK rats. Unlike activin A, CnP attenuated activation of PSC in culture and reduced the production of collagen and MCP-1. In addition, orally administered CnP significantly reduced islet fibrosis in GK rats. These results suggest the efficacy of CnP to inhibit islet fibrosis. Given that CnP also induces differentiation of β -cells, CnP may be a candidate compound to promote regeneration of β -cells in diabetic pancreas.

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References

- Goto Y, Kakizaki M, Masaki N 1975 Spontaneous diabetes produced by selective breeding of normal Wistar rats. *Proc Jpn Acad* 51:80–85
- Kakizaki M, Fujiya H, Goto Y 1979 The pancreatic islets of the spontaneous diabetes rats. *Diabetes J* 7:105–108
- Guenifi A, Abdel-Halim SM, Höög A, Falkmer S, Ostenson CG 1995 Preserved β -cell density in the endocrine pancreas of young, spontaneously diabetic Goto-Kakizaki (GK) rats. *Pancreas* 10:148–153
- Movassat J, Saulnier C, Serradas P, Portha B 1997 Impaired development of pancreatic β -cell mass is a primary event during the progression to diabetes in the GK rat. *Diabetologia* 40:916–925
- Homo-Delarche F, Calderari S, Irminger JC, Gangnerau MN, Coulaud J, Rickenbach K, Dolz M, Halban P, Portha B, Serradas P 2006 Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK Rat. *Diabetes* 55:1625–1633
- Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS 1998 Role of apoptosis in failure of β -cell mass compensation for insulin resistance and β -cell defects in the male Zucker diabetic fatty rat. *Diabetes* 47:358–364
- Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T 1992 Spontaneous long-term hyperglycemia rat with diabetic complications. Otsuka Long-Evans Tokushima Fatty (OLETF) strain. *Diabetes* 41:1422–1428
- Masuyama T, Komeda K, Hara A, Noda M, Shinohara M, Oikawa T, Kanazawa Y, Taniguchi K 2004 Chronological characterization of diabetes development in male spontaneously diabetic Torii rats. *Biochem Biophys Res Commun* 314:870–877
- Clark A, Nilsson MR 2004 Islet amyloid: a complication of islet dysfunction or an aetiological factor in type 2 diabetes? *Diabetologia* 47:157–169
- Zhao HL, Lai FM, Tong PC, Zhong DR, Yang D, Tomlinson B, Chan JC 2003 Prevalence and clinicopathological characteristics of islet amyloid in chinese patients with type 2 diabetes. *Diabetes* 52:2759–2766
- Ehses JA, Perren A, Eppler E, Ribaux P, Pospisilik JA, Maor-Cahn R, Gueripel X, Ellingsgaard H, Schneider MK, Biollaz G, Fontana A, Reinecke M, Homo-Delarche F, Donath MY 2007 Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes* 56:2356–2370
- Friedman SL 2008 Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134:1655–1669
- Watari N, Hotta Y, Mabuchi Y 1982 Morphological studies on a vitamin A-storing cell and its complex with macrophage observed in mouse pancreatic tissues following excess vitamin A administration. *Okajimas Folia Anat Jpn* 58:837–858
- Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS 1998 Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut* 43:128–133
- Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grünert A, Adler G 1998 Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 115:421–432
- Omary MB, Lugea A, Lowe AW, Pandol SJ 2007 The pancreatic stellate cell: a star on the rise in pancreatic disease. *J Clin Invest* 117:50–59
- Ohnishi N, Miyata T, Ohnishi H, Yasuda H, Tamada K, Ueda N, Mashima H, Sugano K 2003 Activin A is an autocrine activator of rat pancreatic stellate cells: potential therapeutic role of follistatin for pancreatic fibrosis. *Gut* 52:1487–1493
- Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I 1996 Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J Clin Invest* 97:1647–1654
- Li L, Yi Z, Seno M, Kojima I 2004 Activin A and betacellulin: effect on regeneration of pancreatic β cells in neonatal streptozotocin-treated rats. *Diabetes* 53:608–615
- Ogata T, Park KY, Seno M, Kojima I 2004 Reversal of streptozotocin-induced hyperglycemia by transplantation of pseudoislets consisting of the β cells derived from ductal cells. *Endocr J* 51:381–386
- Hully JR, Chang L, Schwall RH, Widmer HR, Terrell TG, Gillett NA 1994 Induction of apoptosis in the murine liver with recombinant human activin A. *Hepatology* 20:854–862
- Umezawa K, Hiroki A, Kawakami M, Naka H, Takei I, Ogata T, Kojima I, Koyano T, Kowithayakorn T, Pang HS, Kam TS 2003 Induction of insulin production in rat pancreatic acinar carcinoma cells by conophylline. *Biomed Pharmacother* 57:341–350
- Umezawa K, Ohse T, Yamamoto T, Koyano T, Takahashi Y 1994 Isolation of a new vinka alkaloid from the leaves of *Ervatamia microphylla* as an inhibitor of ras functions. *Anticancer Res* 14:2413–2417
- Ogata T, Li L, Yamada S, Yamamoto Y, Tanaka Y, Takei I, Umezawa K, Kojima I 2004 Promotion of β -cell differentiation by conophylline in fetal and neonatal rat pancreas. *Diabetes* 53:2596–2602
- Fujii M, Takei I, Umezawa K 2009 Antidiabetic effect of orally administered conophylline-containing plant extract on streptozotocin-treated and Goto-Kakizaki rats. *Biomed Pharmacother* 63:710–716
- Apte MV, Park S, Phillips PA, Santucci N, Goldstein D, Kumar RK, Ramm GA, Buchler M, Friess H, McCarroll JA, Keogh G, Merrett N, Pirola R, Wilson JS 2004 Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas* 29:179–187
- Haber PS, Keogh GW, Apte MV, Moran CS, Stewart NL, Crawford DH, Pirola RC, McCaughan GW, Ramm GA, Wilson JS 1999 Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. *Am J Pathol* 155:1087–1095
- Casini A, Galli A, Pignalosa P, Frulloni L, Grappone C, Milani S, Pederzoli P, Cavallini G, Surrenti C 2000 Collagen types synthesized by pancreatic periacinar stellate cell (PSC) co-localizes with lipid peroxidation-derived aldehydes in chronic alcoholic pancreatitis. *J Pathol* 192:81–89
- Ko SH, Hong OK, Kim JW, Ahn YB, Song KH, Cha BY, Son HY, Kim MJ, Jeong IK, Yoon KH 2006 High glucose increases extracellular matrix production in pancreatic stellate cells by activating the renin-angiotensin system. *J Cell Biochem* 98:343–355
- Nomiyama Y, Tashiro M, Yamaguchi T, Watanabe S, Taguchi M, Asaumi H, Nakamura H, Otsuki M 2007 High glucose activates rat pancreatic stellate cells through protein kinase C and p38 mitogen-activated protein kinase pathway. *Pancreas* 34:364–372
- Hong OK, Lee SH, Rhee M, Ko SH, Cho JH, Choi YH, Song KH, Son HY, Yoon KH 2007 Hyperglycemia and hyperinsulinemia have additive effects on activation and proliferation of pancreatic stellate cells: possible explanation of islet-specific fibrosis in type 2 diabetes mellitus. *J Cell Biochem* 101:665–675
- Kikuta K, Masamune A, Satoh M, Suzuki N, Satoh K, Shimosegawa T 2006 Hydrogen peroxide activates activator protein-1 and mitogen-activated protein kinases in pancreatic stellate cells. *Mol Cell Biochem* 291:11–20
- Masamune A, Watanabe T, Kikuta K, Satoh K, Shimosegawa T 2008 NADPH oxidase plays a crucial role in the activation of pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 294:G99–G108
- Andoh A, Takaya H, Saotome T, Shimada M, Hata K, Araki Y, Nakamura F, Shintani Y, Fujiyama Y, Bamba T 2000 Cytokine regulation of chemokine (IL-8, MCP-1 and RANTES) gene expression in human pancreatic periacinar myofibroblasts. *Gastroenterology* 119:211–219
- Masamune A, Kikuta K, Satoh M, Sakai Y, Satoh A, Shimosegawa T 2002 Ligands of peroxisome proliferator-activated receptor- γ

- block activation of pancreatic stellate cells. *J Biol Chem* 227:141–147
36. Klonowski-Stumpe H, Fischer R, Reinehr R, Lüthen R, Häussinger D 2002 Apoptosis in activated rat pancreatic stellate cells. *Am J physiol Gastrointest Liver Physiol* 283:G819–G826
 37. Koyama M, Wada R, Mizukami H, Sakuraba H, Odaka H, Ikeda H, Yagihashi S 2000 Inhibition of progressive reduction of islet β -cell mass in spontaneously diabetic Goto-Kakizaki rats by α -glucosidase inhibitor. *Metabolism* 49:347–352
 38. Atsumi S, Nagasawa A, Koyano T, Kowithayakorn T, Umezawa K 2003 Suppression of TGF- β signaling by conophylline via up-regulation of c-Jun expression. *Cell Mol Life Sci* 60:2516–2525
 39. Reinehr R, Zoller S, Klonowski-Stumpe H, Kordes C, Häussinger D 2004 Effect of angiotensin II on rat pancreatic stellate cells. *Pancreas* 28:129–137
 40. Masamune A, Satoh M, Kikuta K, Suzuki N, Satoh K, Shimosegawa T 2005 Ellagic acid blocks activation of pancreatic stellate cells. *Biochem Pharmacol* 70:869–878
 41. Mizukami H, Wada R, Yonezawa A, Sugawara A, Yagihashi S 2008 Suppression of post-prandial hyperglycemia by pioglitazone improved islet fibrosis and macrophage migration in the Goto-Kakizaki rat. *Diabetes Obes Metab* 10:791–794
 42. Ko SH, Kwon HS, Kim SR, Moon SD, Ahn YB, Song KH, Son HS, Cha BY, Lee KW, Son HY, Kang SK, Park CG, Lee IK, Yoon KH 2004 Ramipril treatment suppresses islet fibrosis in Otsuka Long-Evans Tokushima fatty rats. *Biochem Biophys Res Commun* 316:114–122
 43. Tikellis C, Wookey PJ, Candido R, Andrikopoulos S, Thomas MC, Cooper ME 2004 Improved islet morphology after blockade of the renin-angiotensin system in the ZDF rat. *Diabetes* 53:989–997



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