Promotion of β-Cell Differentiation by Conophylline in Fetal and Neonatal Rat Pancreas

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Conophylline is a vinca alkaloid extracted from the tropical plant *Ervatamia microphylla* and has been shown to induce differentiation of pancreatic AR42J cells. In the present study, we investigated the effect of conophylline on the differentiation of pancreatic precursor cells. In the rat pancreatic rudiment in organ culture, conophylline inhibited the formation of cystic structure and increased the number of insulin-positive cells. Conophylline also markedly increased the expression of mRNA for insulin and the number of pancreatic duodenal homeobox-1–positive cells. These effects of conophylline were similar to those of activin A. We also examined the effect of conophylline on neonatal rats treated with streptozotocin, a model of type 2 diabetes. Treatment with conophylline significantly reduced the plasma glucose concentration and improved glucose tolerance in response to glucose loading. The insulin content and the β-cell mass at 2 months were significantly increased by conophylline. The number of islet-like cell clusters and pancreatic duodenal homeobox-1–positive ductal cells was greater in conophylline-treated rats. These results suggest that conophylline induces differentiation of pancreatic precursor cells and increases the formation of β-cells. *Diabetes* 53: 2596–2602, 2004

Insulin resistance and hyposecretion of insulin are two major features in the pathogenesis of type 2 diabetes (1). Recent studies done in animal models of diabetes have suggested that the β-cell mass is a critical factor in the onset of overt diabetes. For example, insulin receptor substrate-1–deficient mice, made by homologous recombination, have remarkable insulin resistance. Nevertheless, they are not diabetic (2). The amount of β-cell hyperplasia is significant in these mice and perhaps hyposecretion of insulin compensates for the insulin resistance. Consequently, severe insulin resistance per se does not cause diabetes, and the β-cell mass that overcomes insulin resistance can prevent diabetes. In this regard, agents capable of stimulating pancreatic β-cell neogenesis may be potentially innovatory for the treatment of diabetes. This notion is fascinating in light of the fact that regeneration of the β-cell is impaired in animal models of type 2 diabetes. For example, in the Goto-Kakizaki (GK) rat, an animal model of type 2 diabetes, β-cell neogenesis was remarkably impaired (3). Furthermore, augmentation of differentiation potential would have reversed the onset of diabetes. Thus, an administration of glucagon-like peptide 1 or exendin 4 in neonatal GK rats can improve glucose tolerance in adults (4). It is expected that the promotion of β-cell neogenesis can prevent diabetes. Differentiation promoting factors such as glucagon-like peptide 1, exendin 4, islet neogenesis-associated protein (INGAP), betacellulin, hepatocyte growth factor, epidermal growth factor, transforming growth factor-β, and activin have been described (4–9). Among them, activins play a key role in pancreatic development and regulate pancreatic differentiation (10–12). Moreover, activins may regulate pancreatic regeneration (13). Activins, however, have pleiotropic actions in various organs and, in particular, induce apoptosis in some cells (14,15). This action may be considerably deteriorative for clinical use. Agents that reproduce the activin action, but without apoptosis-inducing activity, would be quite useful. In this regard, Umezawa et al. (16) have screened such compounds and recently identified the compound conophylline. Conophylline is extracted from *Ervatamia microphylla*, a tropical plant in Thailand (17). It has a unique structure typical of vinca alkaloid with a molecular weight of 794. As it is smaller than activins, better penetration to the tissues is expected. To elucidate the effect of conophylline on pancreatic progenitor cells, we investigated whether conophylline induces differentiation in fetal pancreas in organ culture. We also examined the effect of conophylline in vivo using neonatal streptozotocin (STZ)-induced diabetic rats.

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ICC, islet-like cell cluster; PDX, pancreatic duodenal homeobox; PP, pancreatic polypeptide; STZ, streptozotocin.

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**RESEARCH DESIGN AND METHODS**

Conophylline was isolated from the leaves of *Ervatamia microphylla* and purified as previously described (18). Briefly, a chloroform extract of the leaves was chromatographed on a silica gel column with hexane-ethyl acetate (1:2) to produce the active fraction. After the active fraction was dissolved in MeOH and the insoluble fraction removed, it was then applied onto a Toyopearl HW-40 column and eluted with methanol to produce the active material. Finally, the crude material was purified by centrifugal partition chromatography and freeze dried from t-butyl alcohol to produce white powder. Recombinant human activin A was provided by Dr. Eto of Ajinomoto (Kawasaki, Japan).
Animals and dissection of pancreatic rudiments. Pregnant Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The morning of the discovery of the vaginal plug was designated as embryonic day 0.5 (E0.5). Pregnant rats at 14.5 days of gestation were killed by cervical dislocation. Embryonic pancreata were removed from the uterus. Embryonic pancreata were dissected from the digestive tracts under a stereoscopic microscope.

Culture of the pancreatic rudiments in three-dimensional collagen gels. Pancreatic rudiments were grown in three-dimensional collagen gels in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mmol/l glutamine, 50 units/ml penicillin, and 75 mg/ml streptomycin. In some experiments, 0.1 μg/ml conophylline was added to the culture medium. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Medium was replaced every 48 h. At the indicated times, the rudiments were photographed and fixed for immunohistochemistry as described below. The DNA content of the rudiment was measured by fluorometric assay (19).

In vivo experiments using neonatal STZ-induced diabetic rats. One-day-old male neonatal Wistar rats received a single intraperitoneal injection of 85 μg/g of streptozocin (STZ) (Wako, Tokyo, Japan) freshly dissolved in 0.05 mol/l citrate buffer (pH 4.5). The day of STZ injection was designated as day 0. The pups were left with their mothers until 4 weeks. All neonates were tested 1 day after the STZ injection (day 1) for blood glucose using Accu-Chek Active (Roche Diagnostics, Mannheim, Germany). The animals were included in this study only if their blood glucose concentration was between 200 and 350 mmol/l. One neonatal STZ-induced diabetic rats were injected subcutaneously with 5 μg/g conophylline or vehicle on days 1, 3, 5, and 7. Fasting blood glucose concentration and body weight were measured daily for subcutaneously with 5 μg/g conophylline or vehicle on days 1, 3, 5, and 7. Fasting blood glucose concentration and body weight were measured daily for the first week and then once a week for up to 8 weeks. The plasma insulin concentration was measured on day 8 and week 8 with an insulin assay kit (Morinaga, Yokohama, Japan), using rat insulin as standard. Eight weeks after the STZ treatment, an intraperitoneal glucose tolerance test (2 g/kg body wt) was performed 1 h fasting. The experimental protocol was approved by the animal care committee of Gunma University.

Immunohistochemistry. The pancreatic rudiments and pancreas were fixed at 4°C in 10% formaldehyde in PBS for 24 h and embedded in Paraplast (Labanord, Templemars, France). Each pancreatic block was serially sectioned by a microtome and collected on multiwell glass slides. One of two consecutive sections was analyzed by immunocytochemistry. The sections were examined and photographed with a microscope (Labonord, Templemars, France). Each pancreatic block was serially sectioned by a microtome and collected on multiwell glass slides. One of two consecutive sections was analyzed by immunocytochemistry. The sections were examined and photographed with a microscope (Labonord, Templemars, France).

RESULTS

Conophylline promoted differentiation of insulin-producing cells in organ culture. Figure 1A shows the macroscopic morphology of pancreatic rudiments cultured in collagen gel for 72 h. The size of the rudiments was almost the same in the control and activin- and conophylline-treated rudiments. The ratio of the DNA content of the control and activin- and conophylline-treated rudiments was 1.0±0.106 (n = 3). At E14.5, the pancreatic rudiments consisted of duct-like epithelia surrounded by a considerable amount of mesenchymal tissue (Fig. 1B, b). A few insulin-positive cells were scattered around the epithelia (Fig. 1B, a). After a 72-h culture, duct-like epithelia expanded and formed cysts (Fig. 1B–D). The number of insulin-positive cells increased slightly (Fig. 1B and C). In activin-treated tissues, expansion of duct-like epithelia was moderate, and insulin-positive cells formed small clusters (Fig. 1B–E). In conophylline-treated tissues, expansion of duct-like epithelia was scarce and many islet-like cell clusters were observed (Fig. 1B–G, h). In addition, formation of small ducts was observed.

Since activin A promotes amylose-secreting AR42J into PP-secreting cells (22), we first measured the changes in the number of PP-positive cells. In specimens obtained at 72 h, the number of PP-, insulin-, or amylose-positive cells was measured. Regarding the number of PP-positive cells, no statistical significance was obtained among the three groups (Fig. 1C). The number of insulin-positive cells in control-treated tissue was significantly lower than that of the control, whereas the number of insulin-positive cells in the conophylline-treated rudiment was significantly greater than that of the control (Fig. 1C).

Activation and conophylline significantly reduced the number of amylase-positive cells. Real-time RT-PCR analysis further confirmed these results. The PP expression of the control group increased fourfold at 72 h, whereas that of the activin- and conophylline-treated groups did not increase significantly (Fig. 2). The insulin expression in the control group increased only slightly. In contrast, the expression of insulin in activin- and conophylline-treated tissues increased ~200-fold at 72 h.

Conophylline promoted early step of β-cell differentiation. We then studied the effect of conophylline in rudiments cultured for a longer period. Conophylline is light sensitive and degraded by ultraviolet rays. We tested various conditions and found that an addition of conophylline every 48 h provided the maximal effect (data not shown). Accordingly, we added conophylline every 2 days and cultured pancreatic rudiments for 10 days. In the control, epithelia expanded and formed cysts and insulin- or PDX-1-positive cells were scattered around them (Fig.
In conophylline-treated tissues, epithelial expansion was scarce and the numbers of insulin- and PDX-1–positive cells increased markedly around duct-like structures (Fig. 3A). Moreover, many ICCs were observed. Quantitative analysis using the National Institutes of Health Image software showed that the relative area for PDX-1- and insulin-positive cells in conophylline-treated tissues were significantly greater than those of the control, and the ratio of area of insulin to PDX-1 in conophylline-treated tissues was lower than that of the control (Fig. 3C).

**Effect of conophylline in neonatal STZ-induced diabetic rats.** We then examined the effect of conophylline in
vivo using neonatal STZ-induced diabetic rats. As shown in Fig. 4A, the plasma glucose concentration peaked on day 2 in neonatal STZ-induced diabetic rats, with a peak value of ~400 mg/dl. The plasma glucose concentration then decreased gradually but remained significantly higher than that in the normal rats at 2 months of age (Table 1). We administered conophylline on days 1, 3, 5, and 7. As shown in Fig. 4A, the plasma glucose concentration was significantly lower on day 2 ($P < 0.01$) and thereafter. At 2 months of age, the plasma glucose concentration was significantly lower in conophylline-treated rats compared with STZ-induced diabetic rats (Table 1). An intraperito-
neal glucose-loading test was done at 2 months. As shown in Fig. 4B, the plasma glucose response to glucose loading was greatly improved in conophylline-treated rats. The insulin secretion in response to glucose loading was also improved in conophylline-treated rats, but the response was still delayed compared with normal rats. As shown in Table 1, the insulin content and the β-cell mass were significantly increased in conophylline-treated rats. Immunohistochemical analysis revealed that the percent of PDX-1-positive ductal cells was markedly increased (Fig. 5A and B), and the number of ICCs was also significantly greater in conophylline-treated rats compared with vehicle-treated rats. Note that conophylline did not affect the number of BrdU-positive ductal cells or BrdU-positive β-cells in the islets (data not shown).

**DISCUSSION**

Here, we demonstrate that conophylline induced differentiation of fetal rat pancreas in organ culture. Previous studies using pancreatic anlage showed that mesenchymal factors promoted differentiation of pancreatic progenitors to exocrine lineage (22), and removal of mesenchymal tissue promoted differentiation to the endocrine lineage (20). Miralles et al. (20) showed that conversion of progenitor cells to endocrine or exocrine cells was modulated by mesenchyme-derived follistatin, an inhibitor of activin A. Collectively, these results imply that differentiation of progenitor cells is regulated by the activin-follistatin system, which acts as an autocrine/paracrine mechanism. In agreement with this notion, activin A induces differentiation of AR42J cells, a model of pancreatic progenitor cells, into endocrine cells (22). Conophylline is also capable of inducing differentiation of AR42J cells into insulin-producing cells. It had been postulated that conophylline reproduces the action of activin A (16). In an organ culture system, conophylline markedly augmented the expression of the insulin gene and significantly increased the number of insulin-positive cells (Figs. 1 and 2). This was also the case in the activin-treated group, which suggests that activin A and conophylline suppressed the effect of mesenchymal cells and promoted differentiation to the endocrine lineage. Because conophylline activates p38 mitogen-activated protein kinase and induces the expression of neurogenin 3, as does activin A (16), conophylline may induce differentiation of pancreatic progenitor cells using the same pathway as activin (16). Hence, conophylline also reproduces the action of activin A in organ culture. Although conophylline increased the number of insulin-positive cells, unlike activin A, it did not significantly induce cystic expansion of the duct-like structure. Instead, there were many small ducts. This indicates that conophylline increased branching morphogenesis of the ducts. It is known that activin A inhibits branching morphogenesis of the pancreatic ducts (14). Conophylline may be less potent in inhibiting branching morphogenesis than activin A. Previous studies by Gittes et al. (23) and Miralles et al. (20) showed that mesenchymal tissue promoted differentiation of pancreatic anlage to the exocrine lin-
eage. On the other hand, typical acinar structure was not formed and the duct-like structures expanded as cystoids in our experiments. This may have resulted from the cultivation conditions of the pancreatic anlage. Because insulin gene expression at 72 h measured by real-time PCR increased remarkably, we stimulated pancreatic anlage with conophylline every 2 days to induce them into insulin-positive cells. As a result, insulin-positive cells increased in the duct-like structures and ICCs invaginated from them. These ICCs were PDX-1 positive, and many of their cells expressed both insulin and PDX-1. These findings suggest that conophylline acts on pancreatic progenitor cells in the duct-like structures and promotes differentiation into insulin-producing cells. Although conophylline increased the number of insulin- and PDX-1-positive cells, the ratio of insulin to PDX-1 was reduced. Conophylline may act on the early stage of β-cell differentiation. In this study, we detected the expression of PP in pancreatic rudiments. This result appears to be contradictory to the generally accepted view that PP is first expressed in the pancreas at birth. However, Herrera and colleagues (24,25) have shown that PP promoter is activated in earlier time points, and PP-positive cells are detected in some studies (26,27). Our result is in line with these reports.

An in vivo study using neonatal STZ-induced diabetic rats further indicates that conophylline promotes differentiation of β-cells and improves glucose metabolism. This model has been widely used to study regeneration of pancreatic β-cells (28–33). Glucagon-like peptide 1 and exendin 4 have been shown to ameliorate diabetes in this model (32). The present results demonstrate that conophylline increased β-cell mass and insulin content and is effective in the promotion of β-cell regeneration. Since conophylline increased the numbers of PDX-1–positive ductal cells and ICCs, it is quite likely that conophylline promotes neogenesis of β-cells by acting on the early stage of β-cell differentiation.

In summary, conophylline reproduces the effect of activin A and acts on pancreatic progenitor cells located in the vicinity of duct epithelium. It promotes differentiation to endocrine cells. Conophylline may be useful for promotion of differentiation of pancreatic β-cells.

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