Administration of Conophylline and Betacellulin-δ4 Increases the β-cell Mass in Neonatal Streptozotocin-treated Rats

Tsutomu KODERA**, Satoko YAMADA*, Yoritsuna YAMAMOTO**, Akemi HARA*, Yuji TANAKA**, Masaharu SENO***, Kazuo UMEZAWA*, Izumi TAKEI** and Itaru KOJIMA*

*Institute for Molecular and Cellular Regulation, Gunma University
**Third Department of Internal Medicine, National Defense Medical College
***Okayama University, Graduate School of Science and Technology
†Department of Applied Chemistry, Faculty of Science and Technology, Keio University Graduate School of Science and Technology
**Department of Internal Medicine, Tokyo Dental College Ichikawa General Hospital

Abstract. The present study was conducted to examine the effect of administration of conophylline (CnP) and betacellulinδ4 (BTCδ4) on the β-cell mass in neonatal streptozotocin-treated rats (neonatal STZ rats). STZ (100 µg/g) was injected into neonatal rats, and then CnP (2 µg/g) and/or BTCδ4 (200 pmol/g) were administered to neonatal STZ rats for 1 week. The plasma glucose concentration was monitored, and an intraperitoneal glucose tolerance test (ipGTT) was performed on day 8 and at 8 weeks after the STZ injection. In neonatal STZ rats treated with control solution (S group), the plasma glucose concentration increased for several days after the STZ injection, returned to nearly normal levels, and then increased gradually after six weeks of age. Eight weeks after the STZ-injection, the plasma glucose concentration was increased significantly compared to that of normal rats. The glucose response to ipGTT was significantly reduced in neonatal sTz rats treated with CnP (CnP group), BTCδ4 (δ4 group) and CnP+BTCδ4 (CnP+δ4 group). The β-cell mass and the insulin content of the pancreas were significantly increased in the CnP group and δ4 group. The effect of CnP+δ4 was greater than that of CnP alone or BTCδ4 alone. CnP+BTCδ4 significantly increased the number of PDX-1-positive ductal cells and the number of insulin/BrdU double-positive ductal cells. These results indicate the efficacy of CnP and BTCδ4 in increasing the β-cells mass of neonatal STZ-treated rats.

Key words: Differentiation, Insulin, Regeneration, Neogenesis

Received Jun. 1, 2009; Accepted Jun. 8, 2009 as K09E-158
Released online in J-STAGE as advance publication Jun. 24, 2009
Correspondence to: Tsutomu KODERA, M.D., Institute for Molecular & Cellular Regulation, Gunma University, Maebashi 371-8512, Japan. E-mail: tom-kode@mte.biglobe.ne.jp

Type 2 diabetes is characterized by relative deficiency of the insulin action due to impaired insulin secretion and concurrent insulin resistance in the target organs [1]. There has been controversy as to whether or not the β-cell mass is actually reduced in patients with type 2 diabetes. Recent studies, however, have clearly shown that the β-cell mass is indeed reduced in patients with type 2 diabetes [2, 3]. Since insulin resistance can be overcome by a compensatory increase in the insulin secretion in animal models of diabetes [4], it is reasonable to assume that expansion of the β-cell mass would improve the morbidity of the type 2 diabetes. Consequently, expansion of the β-cell mass provides a new therapeutic approach to type 2 diabetes. The β-cell mass is determined by a balance between loss and neoformation of β-cells. It is now known that β-cells are formed by either replication of preexisting β-cell or by neogenesis from progenitor cells in the pancreas [5].

We have investigated the action of differentiation factors for β-cells and found that activin A and betacellulin (BTC) act coordinately to facilitate differentiation of β-cells from various types of progenitors [6-8]. Despite their effectiveness both in vitro and in vivo, the two factors have some disadvantages for clinical application. For example, activin A induces apoptosis in certain types of cells when administered in vivo [9]. In addition, BTC is a potent mitogen for various types of
cells including smooth muscle cells [10]. This raises a concern that BTC would promote athelosclerosis when administered in vivo. To overcome these disadvantages, we have been searching for factors devoid of apoptosis-inducing activity or growth-promoting actions. Indeed, we have identified two factors, conophylline (CnP) [11, 12] and BTCδ4 [13]. CnP is a new class of vinca alkaloid which mimics the differentiation-inducing activity of activin A [11, 12]. CnP induces the expression of neurogenin-3 and thereby promotes differentiation of endocrine cells [11]. Unlike activin, CnP does not induce apoptosis neither in vitro and in vivo. BTCδ4 is a splicing variant of the pro-BTC gene. This factor mimics the action of BTC in terms of its differentiation of β-cells. However, because BTCδ4 lacks the epidermal growth factor (EGF) motif, it does not bind to the EGF receptor nor stimulate growth of the target cells. Acting on the different steps of differentiation, CnP and BTCδ4 act coordinately and augment differentiation of β-cells in vitro [14]. In fact, their effect exceeds that of a combination of activin A and BTC [14]. The present study was conducted to assess the in vivo effect of the combination of CnP and BTCδ4 on β-cell differentiation. To this end, we used neonatal rats treated with streptozotocin (STZ), a model of type 2 diabetes [15]. The results show that CnP and BTCδ4 increase the β-cell mass and improve glucose metabolism in neonatal STZ rats.

**Materials and Methods**

**Reagents**

CnP was isolated from leaves of *Ervatamia microphylla* and purified as previously described [16]. Recombinant human BTCδ4 was prepared as described elsewhere [17]. STZ was purchased from Wako Pure Chemicals (Osaka, Japan).

**Animals**

Pregnant Wistar rats (16~18 days of pregnancy) were obtained from Nihon SLC, Hamamatsu, Japan). These rats were caged individually and fed ad libitum with a commercial pellet diet (Nihon CLEA, Tokyo, Japan). They were checked at 0900 for delivery of pups. One-day-old neonates received a single intraperitoneal injection of 100 µg/g body weight of STZ freshly dissolved in 0.1 M citrate buffer (pH 4.5). All neonates were tested one day after the STZ treatment (day 1) for blood glucose using the Freestyle Blood Glucose Monitoring System (Nipro, Japan). The animals were included in the study only when their blood glucose concentrations were above 250 mg/dL on day 1. Five experimental groups were examined; the N group: non-treated normal group, the S group: STZ-injected rats treated with control buffer, the CnP group: STZ-rats treated with CnP, the δ4 group: STZ-injected rats treated with BTCδ4, and the CnP+δ4 group: STZ-rats treated with a combination of CnP and BTCδ4.

One day after the STZ injection, the animals from various litters were randomly placed into five groups. Then, 200 pmol/g body weight of recombinant BTCδ4 or control buffer was injected subcutaneously every day until day 7, and 2 µg/g body weight of CnP dissolved in olive oil was injected intraperitoneally every other day until day 7 according to the experimental groups.

The casual blood glucose concentration and the body weight were measured daily between 1000 to 1200 for the first week and then twice a week for up to 8 weeks.

**Intraperitoneal Glucose Tolerance Test**

On day 8 and at eight weeks after the STZ treatment, an intraperitoneal glucose tolerance test (IPGTT) (2 g/kg body weight) was performed. Blood samples were collected from the tail tips at 30, 60 and 120 min after the injection of glucose. In eight-week-old rats, the test was performed after 14 h fasting.

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

**Tissue Processing**

On day 8, some rats of each group were injected with 1.5 ml of bromodeoxyuridine (BrdU) per 100 g body weight (cell proliferation kit; Amersham Pharmacia Biotech, Little Chalfont, U.K.) and killed 3 h later. The rest of them were anesthetized and decapitated at eight weeks of age. Their pancreata were excised, weighed, and divided into two parts. One portion was fixed at 4°C in 10% formaldehyde in phosphate-buffered saline (PBS) for 24 h and em-
bedded in Paraplast Plus (McCormick Scientific LLC: St. Louis, USA). Each pancreatic block was serially sectioned (5 μm) throughout its length to avoid any bias from regional changes in islet distribution and islet cell composition, and the sections were mounted on slides. At least 10 sections were randomly chosen at a fixed interval throughout the block.

Immunohistochemistry was performed as described previously [18]. In brief, sections were incubated overnight with primary antibodies after 30 min blocking with 1% bovine serum albumin dissolved in PBS. Thereafter, peroxidase-conjugated or biotinylated secondary antibodies were applied for 1 h. We also used horseradish peroxidase streptavidin (HRP, Vector Laboratories) after applying biotinylated antibodies. Staining was visualized by incubation with 3,3′diaminobenzidine-tetra-hydrochloride (DAB) (DAB; Dojin Chemicals, Tokyo, Japan). After staining the nuclei with hematoxylin, sections were mounted in Pristine Mount (Faruma Inc. Tokyo, Japan).

We used primary and secondary antibodies with the following dilutions: polyclonal guinea pig anti-insulin, 1:1000 (DAKO, Carpinteria, CA, USA), polyclonal rabbit anti-human PDX1 antibody, 1:1000 (Transgenic, Kumamoto, Japan), polyclonal rabbit anti-cytokeratin wide spectrum screening, 1:100 (DAKO), alkaline phosphatase-conjugated affinity purified antiguinea pig antibody, 1:200 (CHEMICON, Temecula, CA, USA), biotinylated anti-goat IgG, 1:200(Vector Laboratories). Double staining of BrdU and insulin, or Pdx1 and cytokeratin was performed as described earlier [17].

**Measurement of Plasma Insulin Level**

Plasma insulin was measured by enzyme-linked immunoassay (ELISA) using the ELISA kit (Morinaga, Tokyo, Japan). Immunoreactive insulin was estimated using purified rat insulin as standard.

**Morphometric Analysis**

For quantitative evaluation of the number of islet-like clusters (ICCs) and the β-cell area, we observed insulin stained sections using Image J ver.1.37 image analysis software (National Institute of Health, MD, USA) by means of an AX70 Epifluorescence microscope (Olympus, Tokyo, Japan) with a PXL 1400 cooled charge coupled device camera system (Photometrics, Tucson, AZ, USA) operated with IP Lab Spectrum software (Signal Analysis, Vienna, VA, USA). At least 30 random fields (magnification x 200) from one section (10 sections from different series per block) were measured for the area of all insulin positive cells. The β-cell mass was calculated by multiplying the pancreas weight by the ratio of the β-cell area.

**Measurement of the Insulin Content**

A half portion of the dissected pancreas was homogenized in cold acid-ethanol, heated for 5 min in a 70°C water bath, centrifuged, and the supernatant was then stored at -20°C until insulin assay. Insulin contents were determined by radioimmunoassay (RIA). Immunoreactive insulin was estimated using insulin antibody and rat 125I-monoiodinated insulin (Eiken) as a tracer. These data were adjusted by the weight of the pancreas.

**Statistical Analysis**

Data are expressed as means ± S.E. For comparison between the two groups, an unpaired t-test was used.

**Results**

**Effect of CnP and BTCδ4 on Glucose Metabolism in Neonatal STZ-treated Rats**

STZ was administered one day after birth. On the next day (designated day 1), rats with the plasma glucose concentration higher than 250 mg/dL were studied. Body weight and plasma glucose concentration in the buffer-treated (S group), CnP-treated (CnP group), BTCδ4-treated (δ4 group) and CnP+BTCδ4-treated (CnP+δ4 group) were not different until day 8 (Table 1). A glucose tolerance test was performed on day 8, and the glucose responses were not significantly different in the four groups (Figure 1A). The plasma glucose concentration then decreased in all groups. Six weeks after the STZ-treatment, the plasma glucose concentration then increased gradually. The increments of the plasma glucose were different in the four groups. At eight weeks after the STZ-treatment, the body weight was not significantly different in the four groups, but the plasma glucose concentra-
Histological Analyses of the Effects of CnP and/or BTCδ4

Immunohistochemical analyses were performed using pancreatic sections obtained eight weeks after the STZ treatment. As shown in Figure 2A, islets were disrupted, and small clusters of insulin-positive cells were scarcely observed in the S group (a). In the δ4 group (b), the number of islet-like cell clusters (ICCs) was increased. Likewise, the number of ICCs was increased in the CnP group (c) and some of them were larger in size. In the CnP+δ4 group (d), numerous numbers of ICCs were observed, which were distributed throughout the pancreatic sections. The number of ICCs was counted and the results are shown in Figure 2B. As depicted, the number of ICCs was significantly increased in the CnP+δ4 group. We then calculated the β-cell mass by measuring the β cell area. As shown in Figure 3A, the β-cell mass was increased in
IN VIVO EFFECT OF CnP AND BTCδ4

803

Fig. 2. Morphological Changes in the Pancreas in 8-week-old STZ-treated Rats
A: Pancreatic section in S, δ4, CnP, and CnP+δ4 groups were stained with anti-insulin antibody. Representative micrographs are presented as follows; a: S group. b: δ4 group. c: CnP group. d: CnP+δ4 group. Insulin-positive cells were stained in brown. Nuclei were counterstained with hematoxylin. ICCs around the ducts emerged after the destruction of normal islet structure by STZ. In CnP, δ4 and CnP+δ4 groups, the number and area of ICCs were increased. Black bar shows 200 μm.
B: The number of ICCs in each group. Values are the means ± S.E. (n = 5). *: p <0.05 vs. S group. **: p <0.01 vs. N group.

Fig. 3. Effects of CnP and/or BTCδ4 on the β-cell Mass and the Insulin Content
A: The β-cell mass was calculated in rats of each group at 8 weeks. B: Insulin content was measured in the pancreas of each group at 8 weeks. Values are the mean ± S.E. *: p <0.05 vs. S group.

the δ4 group, the CnP group and the CnP+δ4 group. The effect of CnP was greater than that of δ4 alone, and an additivity was observed among the two treatments. Thus, the effect of CnP+δ4 was significantly higher than that of δ4 alone. The effectiveness of the combination of CnP+δ4 was confirmed by measuring the insulin content (Figure 3B).

To assess the mode of action of CnP and BTCδ4, we analyzed pancreatic sections obtained 8 days after the STZ-treatment. We first analyzed the number of PDX-1-positive ductal cells. Sections were stained with anti-PDX-1 and anti-cytokeratin antibodies. As shown in Figure 4A, in normal rats, PDX-1-positive cells were observed mostly in pancreatic islets. PDX-1/cytokeratin double-positive cells were occasionally observed in the duct. In STZ-treat rats, PDX-1-positive cells were observed in small clusters of endocrine cells. PDX-1/cytokeratin double-positive cells were observed in the duct. In STZ-treated rats administered with CnP+BTCδ4, the number of PDX-1/cy-
sulin/BrdU double-positive cells were observed in the duct (Figure 5A). In STZ-treated rats, most of the islets were disrupted, and insulin/BrdU double-positive cells were observed in the duct. Treatment with CnP+BTCδ4 significantly increased the number of insulin/BrdU double-positive cells. Figure 5B shows the results of quantitative analysis of the number of insulin/BrdU double-positive cells.

tokeratin double-positive cells was increased. Results of quantitative analysis of the number of PDX-1/cytokeratin double-positive cells are shown in Figure 4B.

We then analyzed the replication of insulin-positive cells. Pancreatic sections obtained on day 8 were stained with anti-insulin and anti-BrdU antibodies, and insulin/BrdU double-positive cells were analyzed. In normal rats, insulin/BrdU double-positive cells were observed mostly in islets. In addition, some insulin/BrdU double-positive cells were observed in the duct (Figure 5A). In STZ-treated rats, most of the islets were disrupted, and insulin/BrdU double-positive cells were observed in the duct. Treatment with CnP+BTCδ4 significantly increased the number of insulin/BrdU double-positive cells. Figure 5B shows the results of quantitative analysis of the number of insulin/BrdU double-positive cells.
**Discussion**

In the present study, we evaluated the *in vivo* effect of the combination of CnP and BTCδ4 on neogenesis of β-cells using neonatal STZ-treated rats as a model of type 2 diabetes. The neonatal STZ-treated rat is a model of type 2 diabetes induced in rats with normal genetic background [15]. Our protocol is designed to focus on the effect of various factors on neogenesis of β-cells [15]: CnP and/or BTCδ4 were administered for a week after the treatment with STZ. In this period, neogenesis of β-cells is quite active, and moreover, β-cells present on the day of birth were mostly destroyed by STZ. Therefore, most of the effects induced by CnP and/or BTCδ4 may have been due to stimulation of neogenesis of β-cells from progenitors located in the pancreatic duct rather than replication of pre-existing β-cells. The present results clearly show that both CnP and BTCδ4 are effective and improved glucose metabolism in neonatal STZ-treated rats. This effect was due largely to the increase in the β-cell mass as shown in Figures 2 and 3. Both BTCδ4 alone and CnP alone were effective, and an additivity was observed between the effects of two differentiation factors. The results of the previous *in vitro* study indicate that the site of action of CnP and BTCδ4 is different. For example, CnP acts on the progenitor cells and converts them to endocrine cells by inducing the expression of neurogenin-3, a critical gene for endocrine determination [14, 19]. In contrast, BTCδ4 acts on cells already committed to endocrine lineage and converts them to insulin-producing cells [14]. Given that the two factors act on different steps of β-cell differentiation, it is thus reasonable to conclude that the effects of CnP and BTCδ4 are additive. Figure 5A shows that a combination of CnP and BTCδ4 increased the number of insulin/BrdU double-positive cells. The results, however, do not indicate that the combination of two factors stimulates replication of preexisting β-cells. As depicted in Figure 5A, most of the insulin/BrdU double-positive cells are located in the vicinity of the pancreatic duct. Hence, the combination of CnP and BTC-δ4 accelerates differentiation of progenitors in or by the duct, and newly formed β-cells may be still active in terms of replication.

Although we have shown that the combination of CnP and BTCδ4 increased the β-cell mass and improved glucose metabolism, the effects of these two factors are only modest. Both the β-cell mass and the insulin content in treated rats are much less than those of normal rats, being roughly 50% of those in the normal rats. Obviously, we administered only a limited amount of factors for only a limited period (a week) because of the limited amount of reagents available. Given that these two factors are rather short-acting [20], administration of higher doses of differentiation factors for longer periods would result in a more beneficial effect [20]. This possibility should be examined in the near future. It is also possible that additional factor(s) are needed to completely reverse diabetes. In this regard, the size of ICCs observed in STZ-treated rats is much smaller than that in normal islets. Treatment with CnP and BTCδ4 actually increased the number and the size of ICCs, but still their size was smaller than that of islets in normal rats. This observation implies that the formation of small β-cell clusters is augmented effectively, but they do not appropriately to grow regular-sized islets. This raises a possibility that something is still missing to enlarge ICCs. An additional factor(s), which enlarges ICCs to form well-developed islets, may be required. Further studies are needed to improve the formation of normal-sized islets.

In summary, both CnP and BTCδ4 improved glucose intolerance by increasing the β-cell mass in neonatal STZ-treated rats, and the effects of CnP and BTCδ4 were additive. These factors increased the β-cell mass by promoting neogenesis of β-cells from progenitors in or by the pancreatic ducts.

**References**


3. Ritzel RA, Butler AE, Rizza RA, Veldhuis JD, Butler


