Effects of Kampo medicine, Keishi-ka Shakuyaku-to (TJ-60) on alteration of diacylglycerol metabolism in gastrointestinal smooth muscle of diabetic rats

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ABSTRACT

AIM: To examine the effects of Kampo medicine, Keishi-ka-Shakuyaku-to (TJ-60) on the signal transduction in diabetic gastrointestinal dysfunction. METHODS: Experimental diabetic models were prepared using streptozotocin (STZ)-treated Wistar rats. Randomly selected STZ rats were treated with insulin (12 U·kg⁻¹·d⁻¹) or TJ-60 (1% of food intake). Diacylglycerol (DG) and DG kinase activities were quantified in isolated aortic smooth muscle tissue. RESULTS: One of the key element of the PI-turnover, DG kinase activity in resting state in gastric smooth muscle was significantly increased compared to the control value, and carbachol (CCh)-induced response was not detectable, but it was detected in the control rats. On the other hand resting activity in ileum did not differ from the control, but the CCh-induced responses were suppressed. Treatment with TJ-60 indicated resistant effects for the alteration of DG kinase activities in diabetic intestinal tissues. In order to reveal the mechanism of the effects, total content of DG was measured, because the DG plays important role in the PI-turnover and the DG converted from not only PI but also incorporated glucose under high glucose condition. Patterns of the change in DG levels were similar to those in DG kinase. These results indicate that the effect of TJ-60 occurs at the cellular level of DG. CONCLUSION: Dysfunction of gastrointestinal smooth muscle in diabetes is mediated by an alternation of DG and DG kinase. TJ-60 influences the alteration and relief the dysfunction.

INTRODUCTION

Dysfunction of smooth muscle contractility in diabetes mellitus (DM) has been reported to be associated not only with neuronal factors but also with intra-cellular signaling pathways. However, the detailed mechanisms are not clearly understood.

We have previously reported that the vascular and non-vascular smooth muscle contractility increased in insulin-dependent diabetic mellitus (IDDM) model, streptozotocin (STZ)-induced diabetic rats and we suggested that this dysfunction involving an acceleration of phosphatidylinositol (PI)-turnover was mediated by a hyper-reactivity of diacylglycerol (DG) kinase. Although it was pointed out that a normaliza-
tion of intracellular signaling system may contribute to relief of diabetic complications, the critical targets and therapies were not sufficiently established.

Kampo medicine, a traditional Japanese herbal medicine, Keishi-ka-Shakuyaku-to (TJ-60), when administrated orally for the treatment of hyper-reactive contraction of gastrointestinal smooth muscle has been identified as an effective drug and is widely used for therapy in such patients[9]. In this study, we focused on effect of TJ-60 as a Kampo medicine for gastrointestinal disorder, because the medicine has multiple and unknown actions. The aim of this study is to examine the mechanisms contributing to the dysfunction of gastrointestinal smooth muscle tissue in diabetes and understand the effect of TJ-60 on diabetic dysfunction.

MATERIALS AND METHODS

Materials Carrier- and HCl-free radioactive inorganic phosphate ([13]Pi) was purchased from Du-Pont-New England Nuclear (Boston, MA, USA). STZ, carbachol (CCh), norepinephrine (NE), atropine (Atr), phenoxy benzamine (Phen), and neomycin (Neo) were purchased from Sigma Chemical Co (St Louis, MO, USA). Dioctanoyl glycerol (diC8) was obtained from Avanti Polar Lipids Inc (Alabaster, AL, USA). Biologically synthesized human insulin (insulin) was obtained from Novo Nordisk Pharma (Tokyo, Japan). Keishi-ka-shakuyaku-to (Gui-Zhi-Jia-Shao-Yao-Tang; TJ-60, Lot 250060010) was kindly provided by Tsumura & Co (Tokyo, Japan) as a spray-dried powder product form. Ratio of respective crude drug component was as follows: Peony root (root of Paeony lactiflora Pall) (6.0 g), Cinnamon bark (bark of Cinnamomum cassia BL) (4.0 g), Jujube (fruit of Zizyphus jujuba MILL var inermis REHD) (4.0 g), Glycyrrhiza (root and stolon of Glycyrrhiza glabra L, Glycyrrhizauralensis FISCH) (2.0 g) and Ginger (rhizome of Zingiber officinale ROSC) (1.0 g). The yield of extract was 22 %. The extract contained glycyrrhizin (1.22 %) and paonolin (1.78 %).

Preparation of experimental diabetic rats Experimental diabetes was induced in rats by treatment with STZ[10]. STZ at 60 mg/kg body weight in citrate buffer was injected into the lateral vein of 8-week-old Wistar rats (male, 220-260 g body weight). Age-matched controls were injected with citrate buffer. Rats were decapitated 6-7 weeks after injection. During the period, randomly selected diabetic rats were treated with insulin or TJ-60. In the TJ-60-treated STZ rats, TJ-60 was added to feed as 1% of 1-d intake for 2 weeks. Another selected diabetic rats received insulin subcutaneously daily following confirmation of the development of diabetes 35 d after injection of STZ (insulin-treated diabetics) as described below. On each of the first four days, 9.6 U·kg⁻¹·d⁻¹ of insulin was injected; subsequently, treatment dosage was increased to 12 U·kg⁻¹·d⁻¹. Blood glucose level (110-150 mg glucose/dL in control, 4000≤ mg glucose/L in diabetic rats) was determined on a Tidex glucose analyzer (Bayer-Sankyo, Tokyo, Japan).

Preparation of smooth muscle tissues Rats were killed in an ether-saturated chamber and then smooth muscle tissues were replaced as described previously. Following resection, fat adhesion and connective tissues were removed from the tissues. Internal surface of gastric smooth muscle tissue (mucosa) was removed. Internal surfaces of the aorta were gently rubbed with a wooden rod to eliminate the inhibitory influence of vascular endothelial cells. Tissues were then equilibrated in physiological salt solution (PSS) supplemented with (in mmol/L) NaCl 118, KCl 5.8, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.4, NaHCO₃ 21.4, and glucose 11.1 aerated with 95 % O₂ and 5 % CO₂ at 37 ºC.

Assay of DG kinase activity In order to measure the DG kinase activity, we used a cell permeable species of short-chain DG, diC8 as an exogenous substrate as previously described[11]. DiC8 penetrated to the cell membrane and then [32]Pdioctanoyl-phosphatic acid ([13]P)diC8-PA) was formed by DG kinase. As diC8 did not originally exist in the tissue, we considered that the changes in [32]PdiC8-PA accumulation reflected changes in DG kinase activity.

Measurement of total mass of DG Isolated tissues were incubated in PSS containing various compounds at 37 ºC. The total mass of DG in each tissue was measured as described previously[12]. Diorelin was used as a standard. Results were expressed as mg/g wet weight tissue.

Data analysis The data were expressed as mean ±SEM. Statistical differences were determined by the one-way analysis of variance (ANOVA) followed by the Bonferroni t-test for multiple comparisons.

RESULTS

Basic tests in experimental diabetic rats Basic conditions were checked in diabetic rats at seven weeks
Since diC8 was not originally present in the tissue, we was formed by DG kinase in the cell membrane; subsequently, the level of DG kinase activity in tissues. DiC8 pen- DG, was utilized as an exogenous substrate to measure previously, diC8, a cell-permeable species of short-chain ing radiolabeled DG as an exogenous substrate. As a result, DG kinase activity cannot be measured us- secondly, DG does not penetrate the cell membrane. cannot be estimated from endogenous PA levels. of DG by DG kinase; consequently, DG kinase activity thesis from lysophosphatidic acid and phosphorylation of phosphatidylcholine by phospholipase D,

PA is formed via several routes, including hydrolysis of endogenous PA levels. In the TJ-60-treated rats, body weight was not affected (99.6 % of STZ rats) and another factors including blood glucose level indicated slight and not significant decreases.

In our experimental models of rats, the smooth muscle tissue weights were not significantly different from readings obtained in control rats (data not shown).

Changes in $^{32}$P-diC8-PA accumulation in smooth muscle tissue Recently, findings were reported regarding DG kinase employing biochemical methods. However, several difficulties exist which are associated with the examination of DG kinase activity in intact tissue. Problems arising from measurement of cellular DG kinase activity are as follows. First, PA is formed via several routes, including hydrolysis of phosphatidylcholine by phospholipase D, de novo synthesis from lysophosphatidic acid and phosphorylation of DG by DG kinase; consequently, DG kinase activity cannot be estimated from endogenous PA levels. Secondly, DG does not penetrate the cell membrane. As a result, DG kinase activity cannot be measured using radiolabeled DG as an exogenous substrate. Previously, diC8, a cell-permeable species of short-chain DG, was utilized as an exogenous substrate to measure the level of DG kinase activity in tissues. DiC8 penetrated the cell membrane; subsequently, $^{32}$P-diC8-PA was formed by DG kinase in $^{32}$Pi-pre-labeled tissue. Since diC8 was not originally present in the tissue, we considered the changes in $^{32}$P-diC8-PA accumulation to reflect changes in DG kinase activity.

CCh-induced $^{32}$P-diC8-PA accumulation in gastric smooth muscle tissue In control rats, treatment with 10 µmol/L CCh induced an increase in $^{32}$P-diC8-PA accumulation from the resting value (2.3±0.4 to (5.9±0.6) cpm/mg wet weight tissue) (Fig 1). This CCh-induced increase in the $^{32}$P-diC8-PA accumulation was suppressed by each pretreatment of muscarinic receptor antagonist (1 µmol/L Atr) and DG kinase inhibitor (5 µmol/L R59022). In the STZ rat, the resting level of $^{32}$P-diC8-PA accumulation was significantly enhanced (6.62±0.22 cpm/mg wet weight tissue) to a level similar to the maximal response in CCh stimulation in control rat. This basal accumulation of $^{32}$P-diC8-PA in STZ rat had not responded by the 10 µmol/L CCh stimulation[ (7.2±0.6) cpm/mg wet weight tissue]. Although pretreatment of R59022 suppressed the $^{32}$P-diC8-PA accumulation under resting and CCh-treated conditions, Atr did not affect the responses [(6.2±1.0) cpm/mg wet weight tissue]. In the insulin-treated STZ rat, resting level of the $^{32}$P-diC8-PA accumulation was significantly reduced. The value was 40.0 % of the enhancement in STZ rat. Treatment of CCh slightly increased the accumulation to the level in CCh-treated control rat [5.6±1.0 cpm/mg wet weight tissue]. Pretreatment of Atr and R59022 slightly reduced the response. In the TJ-60-treated STZ rat, the resting level of the $^{32}$P-diC8-PA accumulation was (2.2±0.7) cpm/mg wet weight tissue and the enhancement in STZ rats was completely suppressed (-2.1 % of the enhancement in STZ rat). Moreover, CCh-induced a significant increase in $^{32}$P-diC8-PA accumulation [(5.4±0.6) cpm/mg wet weight tissue]. Inhibitory effects of Atr and R59022 were also detected in the TJ-60-treated STZ rats.

CCh-induced $^{32}$P-diC8-PA accumulation in il-
Using the same strategy in the measurement of gastric smooth muscle, CCh-induced changes in $[^{32}P]$diC8-PA accumulation were measured in ileum (Fig 2). A similar increase of CCh-induced response was detected in control rat ileum [from the resting value (3.2±0.8) to (7.2±0.7) cpm/mg wet weight tissue]; Atr and R59022 also inhibited the responses. Although the resting level was not different from the value in control rats, CCh-induced increase in $[^{32}P]$diC8-PA accumulation in diabetic rats had not been detected [(4.0±0.4) cpm/mg wet weight tissue]. Treatment with Atr or R59022 did not affect the basal level in STZ rats. In STZ rats, insulin treatment had no effect. However, significant increase in $[^{32}P]$diC8-PA accumulation was detected without affecting resting level in TJ-60-treated STZ rat ileum [from resting value of (3.7±0.6) to (8.1±0.7) cpm/mg wet weight tissue]. Inhibitory effects of Atr and R59022 were observed [(4.2±0.7) to (3.7±0.7) cpm/mg wet weight tissue].

**NE-induced $[^{32}P]$diC8-PA accumulation in aortic smooth muscle tissue**

In order to examine whether the TJ-60 effects are specific for gastrointestinal smooth muscle, same measurements of $[^{32}P]$diC8-PA accumulation were performed in aortic smooth muscle tissue...
as a typical vascular smooth muscle (Fig 3). NE (10 μmol/L) induced significant increase in [\(^{32}\)P]diC8-PA accumulation in control rats [from resting value of (5.34±0.25) to (11.2±0.5) cpm/mg wet weight tissue]. In STZ rat aorta, the resting level was significantly reduced [(1.34±0.22) cpm/mg wet weight tissue] and NE-induced responses were greater than the response in control rat [(13.9±0.5) cpm/mg wet weight tissue]. This hyper-reactivity of [\(^{32}\)P]diC8-PA accumulation in STZ rat were recovered by the insulin treatment [from the resting value of (3.6±0.3) to (11.1±0.4) cpm/mg wet weight tissue]. In TJ-60-treated STZ rat, hyper-reactivity of [\(^{32}\)P]diC8-PA accumulation were detected as similar as STZ rat [from resting value of (2.0±0.4) to (13.3±0.5) cpm/mg wet weight tissue].

In all groups of rat aortae, inhibition of \(\alpha\)-receptor by 1 μmol/L phenoxybenzamine (Phen) suppressed NE-induced responses. DG kinase inhibitor, R59022 also inhibited the accumulation.

**Changes in total mass of DG in gastrointestinal smooth muscle tissue** Total content of DG levels were investigated as a key element of PI-turnover in intestinal smooth muscle (Fig 4). In gastric smooth muscle tissue, resting level was (186±6) ng/mg wet weight tissue. This value significantly increased by 10 μmol/L CCh stimulation (286±5) ng/mg wet weight tissue.
On the other side, resting level in STZ rat was significantly greater than control rat [(265±12) ng/mg wet weight tissue], but CCh did not cause further increase [(277±5) ng/mg wet weight tissue]. Treatment of insulin did not affect the total content of DG in resting and CCh-stimulation. However, TJ-60-treatment reduced the resting level compared with diabetic rats but significantly increased the total content of DG after CCh treatment [from the resting value (178±6) to (279±6) ng/mg wet weight tissue].

In ileum, resting level of the total content of DG was not different between different models of rat [(128±5) ng/mg wet weight tissue in control rat]. CCh (10 µmol/L)-treatment significantly increased the DG level in control rat [(219±10) ng/mg wet weight tissue], but the value was slightly reduced in STZ rat. In insulin-treated STZ rats DG level was also inhibited, but in TJ-60-treated rats it was significantly increased as similar to control rat after CCh stimulation [(209±14) ng/mg wet weight tissue].

Effect of phospholinase C (PLC) inhibitor, Neo on the total content of DG in gastric smooth muscle tissue was investigated (Fig 5). Pretreatment with 10 µmol/L Neo slightly reduced the resting DG level [(144±7) ng/mg wet weight tissue] and CCh-induced increase was completely suppressed [(163±9) ng/mg wet weight tissue] in control rat. In STZ rat, enhanced resting DG level was not influenced by the Neo treatment [(249±11) ng/mg wet weight tissue] and CCh-response was also unaffected [(241±17) ng/mg wet weight tissue].

### DISCUSSION

The present study confirms that alternations of PI-turnover involving both DG and DG kinase are associated with dysfunction of gastrointestinal smooth muscle in diabetes. Our findings suggest that TJ-60-treatment causes a normalization of the intracellular signaling pathway and it may relieve gastrointestinal complications in diabetes.

We have previously reported that the diabetic dysfunction of smooth muscle involved an alternation of intracellular signaling system\[5\]. Especially, PI-turnover plays a main role in the smooth muscle contraction\[14,15\]. In the gastric smooth muscle, enhanced contractility has been reported in diabetes and it was accompanied by enhanced protein kinase C (PKC) activity\[8\]. This uncontrolled hyper-reactivity may lead to dysfunction of gastrointestinal tissue as a diabetic complication. Actually, diabetic patients appear to be particularly prone to disorder of intestinal tissue. However, detail mechanism of the alternation of intracellular signaling system in diabetes and effective therapy has not been established. In this study, we focused a TJ-60, as one of the herbal medicines for intestinal disorders, because TJ-60 has multiple and unknown effects on intestinal tissue\[9 \].

In our STZ-induced experimental diabetic model, typical changes in basal parameters (body weight, water drunk, volume of urine and food intake) were observed (Tab 1 and not shown data)\[16, 17\]. The blood glucose levels in STZ rats always maintained at a submaximal level, this value did not depend on food intake. Following insulin treatment, almost all parameters including blood glucose level were restored to the normal values as seen in control rats. Thus, it was confirmed that the STZ rat was an IDDM model. In the TJ-60-treated rats, however, most of the parameters were not affected by the treatment and TJ-60 had no effect on blood glucose.

In gastric and ileum smooth muscle tissues, CCh induced increases in DG kinase activity mediated by muscarinic receptor stimulation in control rats (Fig 1, tissue).
It was confirmed by Atr and R59022 pre-treatment. However, DG kinase activity had significantly altered at resting state in STZ rats. Submaximal enhancement of the DG kinase activity was observed in gastric smooth muscle not in ileum smooth muscle. Although, resting levels of DG kinase in both tissues were different, treatment of CCh did not affect the DG kinase activities. The fact that Atr did not influence the DG kinase activities in STZ rats suggests that sensitization of muscarinic receptors may not be involved. In gastric smooth muscle tissue isolated from TJ-60-treated STZ rats, the resting level of the DG kinase activity returned to the level in control rat and CCh-induced response was also observed. On the outer side, DG kinase activity in TJ-60-treated rat ileum had not altered in resting state, but the activity was significantly increased by CCh stimulation. These results indicated that the TJ-60 influenced the alteration of DG kinase activity in stomach and intestinal tissues. It is not clear whether the alternation of DG kinase activities reflects the contrasting patterns in digestive tract; however, the interesting point is that TJ-60 normalized both patterns of changes in DG kinase activity in gastrointestinal smooth muscle tissues. We speculate that the normalization of DG kinase may represent a therapeutic effect of TJ-60 on the disorder of intestinal tissue in diabetes.

We next investigated mechanisms of the DG kinase alternation in diabetes and the effects of treatment with TJ-60. Regulatory factors of an intracellular DG kinase have been suggested. We also reported that both intracellular calcium and PKC could modulate the DG kinase activity [11]. An increased cellular DG level activates the PKC [18] which in turn phosphorylates and directly activates the DG kinase [19, 20]. In this regulation, phosphorylation of DG by DG kinase is dependent on intracellular calcium and plays as a feed back mechanism for reduced cellular DG level and contributes to an acceleration of PI-turnover. We therefore thought that the DG level was an important element in the alternation of DG kinase in diabetes and measured the total content of DG in intestinal smooth muscle. It is widely accepted that the cellular source of DG was derived largely from phosphatidilinositol by PLC in normal condition [19, 22], except for that a part of incorporated glucose was converted to DG not mediated by PLC-pathway under high-glucose condition [23, 24]. In gastric and intestinal smooth muscle tissues in control rats, the total content of DG was significantly increased by CCh stimulation (Fig 4) and was inhibited by PLC inhibitor (Fig 5). In the STZ rat, significant enhancement of resting DG level in gastric smooth muscle and suppressed CCh response in ileum were observed. Although treatment with insulin did not affect these responses, treatment with TJ-60 reached the values in control rats. These patterns of changes were matched to the response in DG kinase activity in control and STZ rats. We considered that the TG-60 influenced the formation of DG from incorporation and/or conversion from glucose under high-glucose condition like diabetes. It might induce changes in PI-turnover mediated by DG kinase activity. This is also supported by the fact that pre-treatment with Neo did not affect the resting and CCh-treated DG levels in gastric smooth muscle (Fig 5). The exact relationship between TJ-60 and cellular DG level is not clear, but it was suggested that TJ-60 may influence one of the steps in the conversion of glucose to DG, because TJ-60 effects were not followed by reduction of blood glucose level (Tab 1) and they are larger than insulin effects (Fig 1, 2).

Finally, we investigated the effects of TJ-60 on vascular smooth muscle contraction (Fig 3) and found hyper-reactivity of DG kinase in aorta [35], but TJ-60 had no effects. These results indicated that the normalizing effect of TJ-60 on the alternation of DG and DG kinase activity in diabetes was relatively selective to gastrointestinal smooth muscle.

In conclusion, we have confirmed that the disorder of gastrointestinal smooth muscle in diabetes is associated with an alteration in PI-turnover involving changes in both DG and DG kinase activity and we found that treatment with the Japanese traditional medicine TJ-60 had a selective normalizing effect on the contents of DG and DG kinase in gastrointestinal smooth muscle and it may contribute to a relieve of intestinal complications in diabetes.

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