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European Journal of Pharmacology 426 (2001) 201–206

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Effects of bezafibrate on β-cell function of rat pancreatic islets

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Received 26 February 2001; received in revised form 6 July 2001; accepted 10 July 2001

Abstract

Bezafibrate is an activator of peroxisome proliferator-activated receptors (PPAR) α. The present study was performed to investigate the effects of bezafibrate and the PPAR α activator, 4-Cholro-6-(2.3-xylidino)-2-pyrimidin-ylthio acetic acid (WY14643), on the β-cell function of rat pancreatic islets in vitro. In islets cultured with 300 μM bezafibrate or WY14643 for 8 h, a low glucose concentration induced insulin release and increased the levels of mRNA for PPAR α, acyl CoA oxidase, carnitine palmitoyl transferase-1, pyruvate dehydrogenase E1 α or pyruvate carboxylase. In contrast, after a 48-h culture period, a high glucose concentration induced insulin release and islet insulin content, but decreased the levels of mRNA for glucose transporter-2 (GLUT-2), preproinsulin or pancreatic/duodenal homeobox-1. Diazoxide, the KATP channel opener, restored these responses. We conclude that bezafibrate enhances insulin release through the activation of PPAR α gene expression during a short culture period, whereas it may contribute to β-cell dysfunction through the mechanism of "excessive stimulation" during longer culture periods.

Keywords: Bezafibrate; PPAR α (peroxisome proliferator-activated receptor); Pancreatic/duodenal homeobox-1; Diazoxide; β-Cell dysfunction

1. Introduction

Bezafibrate has clinically been used to improve lipid metabolism, especially by reducing triglyceride levels. It appears to reduce the transcription of apo-C III, an inhibitor of lipoprotein lipase activity, and increases lipoprotein lipase activity, thus lowering the concentration of total and very low density lipoprotein triglyceride (Auwerx et al., 1996). Several hypolipidemic fibrates, such as bezafibrate, clofibtare, gemfibrate and fenofibrate, are known to activate peroxisome proliferator-activated receptors (PPAR) α. PPAR is a member of the steroid hormone receptor superfamily (Issemann and Green, 1990) and three types of PPARs (PPAR α, PPAR δ and PPAR γ) have been described in rodents and humans. PPAR α is primarily expressed in the liver and regulates aspects of lipoprotein metabolism (Staels et al., 1998). PPAR γ can bind thiazolidinediones, which have been shown to enhance muscle glucose consumption, reduce hepatic glucose production and improve insulin resistance (Nolan et al., 1996; Spiegelman, 1998). PPAR isoforms are also expressed in islets (Braissant et al., 1996), in which their roles are unknown. It is reported that bezafibrate reduces the blood glucose level as well as the serum triglyceride level in patients with type-2 diabetes mellitus (Ogawa et al., 2000). Some evidences for effects of fibrates effects on insulin sensitivity has been reported (Riccardi et al., 1989; Avogaro et al., 1995).

The present study was performed to investigate the effects of bezafibrate on insulin release and the gene expressions of enzymes involved in insulin synthesis or lipid metabolism in rat pancreatic islets. Transcription factors, such as PPAR α or pancreatic/duodenal homeobox-1, were found to be a key molecules in the effects of bezafibrate on β-cell functions.

2. Material and methods

2.1. Materials

Glucose, L-arginine, Dulbecco's Modified Eagle Medium (DMEM), histopaque and diazoxide were purchased from Sigma (St. Louis, MO). Bezafibrate was from Kissei

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01204-3

Chemical (Matsumoto, Japan). 4-Chloro-6-(2.3-xylidino)-2-pyrimidin-ylthio acetic acid (WY14643) was from Cayman Chemical (MI, USA). Rat insulin was from Cosmo Bio (Tokyo, Japan). Collagenase was from Boehringer Mannheim (Mannheim, Germany). Penicillin, streptomycin and fetal calf serum were from Life Technology (Grand Island, NY). The insulin radioimmunoassay (RIA) kit was from Kabi Pharmacia (Tokyo, Japan). Tissue culture flasks were from Falcon (Plymouth, England).

2.2. Animals

Female Wistar rats (150–250 g body weight) were bred under pathogen-free conditions at the Kyushu University Animal Center, Fukuoka, Japan. They had free access to tap water and standard pelleted chow (Clea Japan Tokyo, Japan). They were exposed to a 12-h light (0600–1800 h), 12-h dark cycle. All experiments were approved by the ethics committee for animal experiments at the Faculty of Medicine, Kyushu University and carried out according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, as well as Law No. 105 and Notification No. 6 of the Japanese Government.

2.3. Isolation of islets

Pancreatic islets were isolated by collagenase digestion as previously described (Lacy and Kostianovsky, 1967). Collagenase was injected into the common bile duct at a concentration of 2 mg/ml in 10 ml Hanks solution. The pancreas was digested at 37° for 20 min. Islets were partly separated from exocrine tissue by gradient centrifugation ($1000 \times g$ 20 min, 4 °Cwith histopaque. Islets were transferred to DMEM containing 5.5 mM glucose, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 10% fetal calf serum and then cultured, free-floating, at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 12 h (primary culture) to remove exocrine or other tissues.

2.4. Islets culture

After the period of primary culture, islets were selected under stereomicroscope and transferred to tissue culture flasks. In some experiments, after primary culture, batches of three islets was incubated with bezafibrate or WY14643 concentrations (3, 30, 300, 3000 μM) for 1 h. Culture was carried out in DMEM containing 5.5 mM glucose with or without 300 μM bezafibrate or 300 μM WY14643 for up to 48 h at 37 °C in a humidified atmosphere of 5% CO_2/95% air. In other experiments, cultures under the same conditions as above were carried out with or without 100 μM diazoxide.

2.5. Insulin release

After each culture period, islets were preincubated at 37 °C for 30 min in Krebs-Ringer bicarbonate (KRB) medium (Umbreit et al., 1957) of the following composition: 143 mM Na^+, 5.8 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 124.1 mM Cl^-, 1.2 mM SO_4^{3-}, and 25 mM CO_3^{2-}, pH 7.4, supplemented with 10 mM HEPES, 0.2% bovine serum albumin and 3.3 mM glucose. After preincubation, islets were selected under a microscope and batches of three islets were transferred to tubes containing 300 μl of KRB with 3.3, 11, 16.8, 27 mM glucose. Final incubations were then carried out at 37 °C for 60 min in a water bath with continuous shaking and under an atmosphere of 5% CO_2/95% air. At the end of the incubation, aliquots of the incubation media were retrieved for assay of insulin concentrations. Islets that had been exposed to 3.3 mM glucose in a final incubation were retrieved for the later determination of the islet insulin content. Insulin was measured by RIA, using rat insulin as standard. For the determination of islet insulin content, three islets were each put into 200 μl of acid-ethanol (0.18 M HCl in 95% ethanol). Insulin was extracted overnight at 4 °C after sonication, as previously described (Grill et al., 1981).

Table 1
Sequence of PCR primer

mRNA	Primer sequences (5′–3′)	mRNA	Primer sequences (5′–3′)
PPAR α	CCT TTT TGT GGC TGC TAT	Carnitine palmitoyl	TAT GTG AGG ATG CTG CTT CC
	TCC CTG CTC TCC TGT ATG	transferase-1	CTC GGA GAG CTA AGC TTG TG
GLUT-2	TTA GCA ACT GGG TCT GCA AT	Acyl CoA oxidase	GCC CTC AGC TAT GGT ATT AC
	GGT GTA GTC CTA CAC TCA TG		AGG AAC TGC TCT CAC AAT GC
Glucokinase	AAG GGA ACT ACA TCG TAG GA	Pancreatic/duodenal	GAG CAG GAT TGT GCC GTA ACC
	CAT TGG CGG TCT TCA TAG TA	homeobox-1	CTC AAA GTT TTC AGA AGC TCG
Hexokinase I	GGC TCA GAG GAG ACC CTT CG	Preproinsulin	TGC CCG GGC TTT TGT CAA AC
	CCA GGT CGA ACT TGA ATC AT		CTC CAG TGC CAA GGT CTG AA
Pyruvate dehydro-	TCA AGT ACT ACA GGA TGA TG	β-actin	CGT AAA GAC CTC TAT GCC AA
genase E1 α	GGC GTA CAT GTG CAT TGA TC		AGC CAT GCC AAA TGT GTC AT
Pyruvate	ACT TGT ATG AGC GGG ACT GC		
carboxylase	TGA CCT TGA CGG GGA TTG GA		

2.6. Measurement of mRNA levels

Total RNA was extracted by the TRIzol isolation method (Life Technologies, Gaithersburg, MD) from about 100 islets isolated from individual rats. The mRNA levels of proteins (described below) were measured by using semi-quantitative polymerase chain reaction (PCR) with 5.5 mM glucose-cultured islets with or without 300 μM bezafibrate or 300 μM WY14643 for up to 48 h. In some experiments, cultures were carried out with 100 μM diazoxide. Total RNA was reverse-transcribed by random priming using Avian Myeloblastosis Virus reverse transcriptase (RT) (first-strand DNA synthesis) according to the manufactur's instructions. One microliter of RT reaction mix was amplified with primers specific for each protein described in Table 1: PPAR α (Inoue et al., 1997); glucose transporter-2 (GLUT-2), glucokinase, hexokinase-I and β-actin (Tokuyama et al., 1995); pyruvate dehydrogenase E1 α (Amessou et al., 1998); pyruvate carboxylase (Melendez et al., 1999); carnitine palmitoyl transferase-1 (Zou et al., 1997); acyl CoA oxidase, pancreatic/duodenal homeobox-1 and preproinsulin (Wang et al., 1998) in total volume of 50 μl. Linearity of the PCR reaction was tested by amplification. The samples were amplified in 18–20 cycles for preproinsulin, 30–32 cycles for GLUT-2, glucokinase, hexokinase-1 (HK), pyruvate carboxylase, pyruvate dehydrogenase E1 α, acyl CoA oxidase, carnitine palmitoyl transferase-1, pancreatic/duodenal homeobox-1, β-actin, and 32–34 cycles for PPAR α using the following condition: 92 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min. Aliquots (10 μl) of the PCR mixture were tested on 1% agarose gels. Gels were stained with ethidium bromide. Signals were quantified by scanning densitometry using NIH Image 1.56 software.

2.7. Statistical analysis

All results are presented as means ± S.E.M. of more than four experiments. Data were analyzed by Student's *t*-test (paired) or one-way analysis of variance (ANOVA) with Fisher's Least Significant Difference test. *P* values less than 0.05 were considered to indicate significant differences.

3. Results

3.1. Insulin release by bezafibrate or WY14643

Insulin release from three islets, which appeared to be almost the same size under stereomicroscope, was measured after incubation with 0–3000 μM bezafibrate or 0–3000 μM WY14643 containing 3.3 mM glucose for 1 h. Insulin release was significantly increased by coincubation with 300 or 3000 μM bezafibrate or 300 μM WY14643 (Fig. 1). Since almost maximal effects on insulin release were obtained with 300 μM of bezafibrate or WY14643, a concentration which was higher than that used in clinical practice (10–30 μM), we used the concentration of 300 μM for the following experiments.

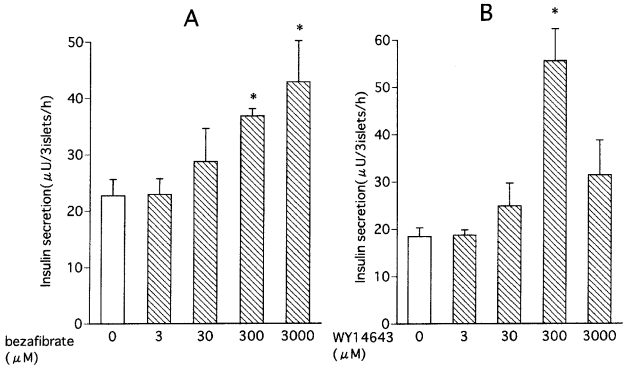

Fig 1. Insulin release induced by 0–3000 μM bezafibrate or WY14643 for 1 h. Panel A represents insulin release induced by bezafibrate. Panel B, insulin release induced by WY14643. Date are expressed as means ± S.E.M. of four experiments. $^{*}P < 0.05$ vs. 0 μM bezafibrate or WY14643.

3.2. Effects of bezafibrate on glucose-induced insulin release or insulin content in cultured islets

After an 8- or 48-h culture period, insulin release induced by 3.3, 11, 16.8 or 27 mM glucose or islet insulin content was measured in islets cultured with 5.5 mM glucose with or without 300 μM bezafibrate. In control islets cultured with only 5.5 mM glucose, neither islet insulin content or insulin release induced by 3.3 to 27 mM glucose changed significantly during 48 h. In islets cultured with 300 μM bezafibrate for 8 h, insulin release induced by 3.3 and 11 mM glucose was significantly increased and that induced by 16.8 and 27 mM glucose was slightly increased compared to that without the agent. The insulin content of islets cultured with 300 μM bezafibrate for 8 h was not changed compared to that of islets cultured without the agent (Fig. 2). However, in islets cultured with 300 μM bezafibrate for 48 h, insulin release induced by 3.3, 16.8 and 27 mM glucose was significantly decreased, but that induced by 3.3 mM glucose did not change. The insulin content of islets cultured with 300 μM bezafibrate for 48 h was also significantly decreased compared to that of islets cultured without the agent (Fig. 3). In order to test the viability of islets cultured with 300 μM bezafibrate, we measured insulin accumulation in the culture medium of islets cultured with or without bezafibrate. Insulin accumulation in culture medium of islets cultured with bezafibrate was higher than that of the medium of islets cultured without the agent for any culture period (data not shown). In order to evaluate the mechanism of the inhibitory effects of bezafibrate on insulin release or content, we tested the effects of diazoxide, an ATP-sensitive K channel opener, on cultured islets. Coculture with

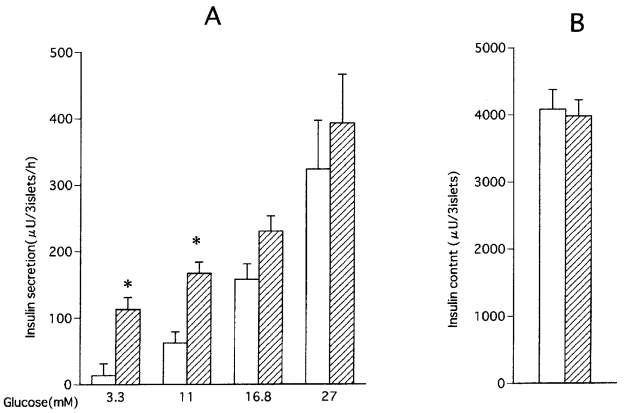

Fig 2. Glucose-induced insulin release and insulin content of islets cultured for 8 h. Panel A represents 3.3, 11, 16.8, 27 mM glucose-induced insulin release. Panel B, islet insulin content. Open bar: islets cultured without bezafibrate. Hatched bar: islets cultured with 300 μM bezafibrate. Date are expressed as means ± S.E.M. of four experiments. * $P < 0.05$ vs. islets without bazafibrate.

Table 2
Quantification of PPAR α, acyl CoA oxidase, carnitine palmitoyl transferase-1, pyruvate dehydrogenase E1 α or pyruvate carboxylase mRNA expression by semiquantitative RT-PCR in islets cultured for 8 h
Means ± S.E.M. of four experiments.

mRNA	Bezafibtare concentration (μM)	mRNA/ β-actin mRNA
PPAR α	0	1.036 ± 0.027
	300	1.286 ± 0.072[a]
Acyl CoA oxidase	0	1.044 ± 0.020
	300	1.235 ± 0.062[a]
Carnitine palmitoyl transferase-1	0	1.023 ± 0.035
	300	1.224 ± 0.045[a]
Pyruvate dehydrogenase E1 α	0	1.052 ± 0.016
	300	1.698 ± 0.222[a]
Pyruvate carboxylase	0	1.038 ± 0.017
	300	1.391 ± 0.111[a]

[a] $P < 0.05$ vs. islets cultured without bazafibrate.

100 μM diazoxide restored the impairment of glucose-induced insulin release or the insulin content of islets cultured with 300 μM bezafibrate (Fig. 3). Similar results were obtained for islets cultured with 300 μM WY14643 for 8 or 48 h (data not shown).

3.3. Gene expression in islets cultured with bezafibrate or WY14643 for 8 or 48 h

In islets cultured with 300 μM bezafibrate for 8 h, mRNA of PPAR α and other enzymes which are related to fat β oxidation (carnitine palmitoyl transferase-1, acyl CoA oxidase) were increased compared to that of islets cultured without bezafibrate (Table 2). After 48 h, however, the enhancement of mRNA expression by bezafibrate disappeared (data not shown). In the same manner, pyruvate dehydrogenase E1 α and pyruvate carboxylase mRNA was increased after 8 h of culture (Table 2) with no change after 48 h. Glucokinase, hexokinase-I, GLUT-2, preproinsulin or pancreatic/duodenal homeobox-1 mRNA in islets cultured with bezafibrate did not change after 8 h with no change even after 48 h in the case of glucokinase, hexokinase-I (data not shown). In contrast, after 48 h of culture, GLUT-2, preproinsulin or pancreatic/duodenal homeobox-1 mRNA was decreased by coculture with bezafibrate. Furthermore, diazoxide restored the impairment of GLUT-2, preproinsulin or pancreatic/duodenal home-

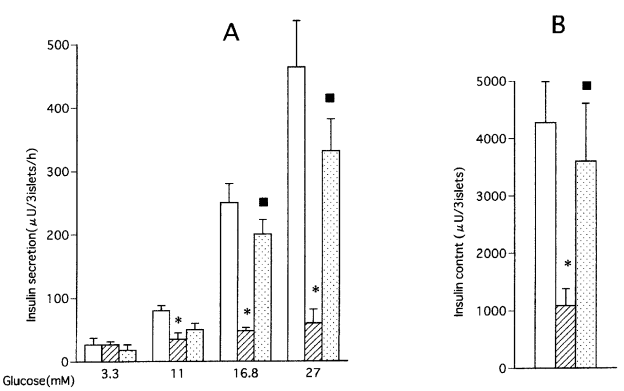

Fig 3. Glucose-induced insulin release and insulin content of islets cultured for 48 h. Panel A represents 3.3, 11, 16.8, 27 mM glucose-induced insulin release. Panel B, islet insulin content. Open bar: islets cultured without bezafibrate or daizoxide. Hatched bar: islets cultured with 300 μM bezafibrate. Dotted bar: islets cultured with both 300 μM bezafibrate and 100 μM diazoxide. Date are expressed as means ± S.E.M. of four experiments. * $P < 0.05$ vs. islets without bazafibrate or diazoxide. ■ $P < 0.05$ vs. islets cultured with 300 μM bazafibrate.

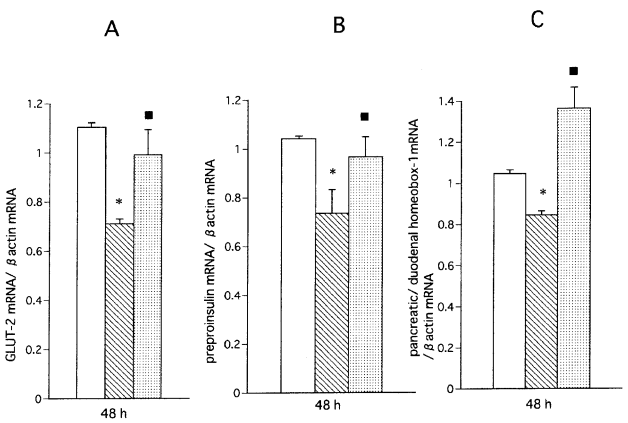

Fig 4. Quantification of GLUT-2, preproinsulin, pancreatic/duodenal homeobox-1 mRNA expression by semiquantitative RT-PCR in islets for 48 h. Panel A represents GLUT-2 mRNA expression. Panel B, preproinsulin mRNA expression. Panel C, pancreatic/duodenal homeobox-1 mRNA expression. Open bar: islets cultured without bezafibrate or diazoxide. Hatched bar: islets cultured with 300 μM bezafibrate. Dotted bar: islets cultured with 300 μM bezafibrate and 100 μM daizoxide. Means ± S.E.M. of four experiments. * $P < 0.05$ vs. islets cultured without bezafibrate or diazoxide. ■ $P < 0.05$ vs. islets cultured with 300 μM bezafibrate.

obox-1 mRNA expression after 48 h of culture (Fig. 4). Similar results were obtained in islets cultured with 300 μM WY14643 for 8 and 48 h (data not shown).

4. Discussion

Bezafibrate is an activator of PPAR α. In the current study, bezafibrate and WY14643 directly stimulated insulin release during a 1-h incubation. However, 3000 μM WY14643 inhibited insulin release compared to the effect of 300 μM of the agent, which implies that 3000 μM of WY14643 might be toxic for islets. These findings indicate that PPAR α activation by itself may stimulate insulin release in islets. We also showed that PPAR α mRNA was increased in islets cultured with bezafibrate or WY14643 for 8 h. The genes encoding enzymes involved in the β-oxidation of free fatty acids (carnitine palmitoyl transferase-1, acyl CoA oxidase) have a PPAR response element (Keller et al., 1993a,b; Kliewer et al., 1994, 1997). In the present study, carnitine palmitoyl transferase-1 mRNA and acyl CoA oxidase mRNA were increased in islets cultured with PPAR α activators for 8 h. In the present study, we showed that pyruvate dehydrogenase E1 α mRNA and pyruvate carboxylase mRNA increased in islets cultured with bezafibrate or WY14643 for 8 h. Both pyruvate dehydrogenase E1 α and pyruvate carboxylase are key molecules in glucose-induced insulin release at the level of pyruvate metabolism (MacDonald et al., 1991) or the pyruvate–malate shuttle (MacDonald, 1995). Although the relationship between PPAR α and pyruvate dehydrogenase E1 α or pyruvate carboxylase is not definite, it is possible that the increase in pyruvate dehydrogenase E1 α or pyruvate carboxylase mRNA may contribute to the enhancement of glucose-induced insulin release in islets cultured with bezafibrate islet for 8 h.

In contrast, the PPAR α activators impaired glucose-induced insulin release or the insulin content of islets when the in culture period was extended to 48 h. PPAR α, carnitine palmitoyl transferase-1 or acyl CoA oxidase mRNA did not change after treatment with the PPAR α activators for 48 h. In these islets, GLUT-2, preproinsulin or pancreatic/duodenal homeobox-1 mRNA was significantly decreased. GLUT-2, the high-Km glucose transporter, plays an important role in the regulation of glucose metabolism, accounting for the impaired insulin release in islets cultured with PPAR α activators for longer period. The decrease in pancreatic/duodenal homeobox-1 mRNA (a major transcription factor of insulin gene) could lead to a decrease in preproinsulin mRNA (Watada et al., 1996a,b; Bonny et al., 1997; Lu et al., 1997). This may be an explanation for the impaired insulin release or content of the treated islets.

It is well known that the diabetic state induces a desensitization that is specific for glucose in pancreatic beta cells (Weir et al., 1986). In in vivo experiments, glucose infusion to nondiabetic rats leads to hyperglycemia and produces glucose desensitization (Leahy et al., 1986). The recovery from hyperglycemia to normoglycemia restores the defects in insulin, pancreatic/duodenal homeobox-1 gene expression and improves insulin release in Zucker diabetic fatty rats ZDF rats (Harmon et al., 1999). Moreover, in in vitro experiments, chronic exposure of β-cell line to supraphysiologically high glucose concentrations decreases insulin gene promoter activity (Olson et al., 1993) and the return to physiological concentrations of glucose restores insulin release and mRNA levels (Robertson et al., 1992). In the present study, we showed that coculture with diazoxide restored the impaired high glucose-induced insulin release or islet insulin content together with GLUT-2, pancreatic/duodenal homeobox-1 or preproinsulin mRNA levels in bezafibrate-cultured islets for 48 h. Diazoxide is a potent inhibitor of insulin release and acts by opening KATP channels. It seems plausible that chronic exposure to bezafibrate impaired insulin release through the "excessive stimulation"mechanism of (Sako and Grill, 1990).

In the present study, we used 300 μM of bezafibtare, a concentration which is much higher than that used in clinical practice (1–10 μM). We observed that in islets cultured with 30 μM bezafibrate for 48 h, high glucose-induced insulin release and islet insulin content did not change. Further investigations, including experiments for longer culture periods with bezafibrate, are necessary to clarify the clinical implications of the PPAR α activator effect on β-cell function.

In conclusion, bezafibrate at supraphysiological concentration enhances insulin release in rat pancreatic islets through the activation of PPAR α gene expression during short culture periods. However, extended exposure of islets to bezafibrate reduces pancreatic/duodenal homeobox-1, GLUT-2 gene expression and leads to β-cell dysfunction, probably through the "excessive stimulation" mechanism.

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