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Fenofibrate, troglitazone and 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ close K_{ATP}-channels and induce insulin secretion.

Kenju Shimomura, Hiroyuki Shimizu, Mizuho Ikeda, Shuichi Okada, Masafumi Kakei, Shigeji Matsumoto, Masatomo Mori

Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine (K.S., H.S., S.O., M.M.); Department of Physiology, Nippon Dental University at Tokyo (M.I., S.M.); Department of Geriatric Medicine, Akita University School of Medicine (M.K.)

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Address correspondence to Kenju Shimomura, MD.

Department of Medicine and Molecular Science,
Gunma University School of Medicine
3-39-15 Showa-machi, Maebashi, Gunma, 371-8511 Japan
Tel: 81-27-220-8501
Fax: 81-27-220-8501
E-mail; kenju.shimomura@physiol.ox.ac.uk

Abbreviations: G, conductance; PPAR, peroxisome proliferator-activated receptors;
 K_{ATP} channel, ATP-sensitive potassium channel; SUR, sulfonylurea
receptor; Kir, inwardly rectifying K^+ channel; VDCC, voltage dependent
 Ca^{2+} channel

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Abstract

It is known that peroxisome proliferator-activated receptor (PPAR) γ ligands stimulate acute-phase insulin secretion with a rapid Ca^{2+} influx into pancreatic β -cells, but the precise mechanisms are not clear. The effects of PPAR α ligands on pancreatic β -cells also remain unclear. We investigated the effects of PPAR α ligands (fenofibrate and fenofibric acid), a PPAR γ ligand (troglitazone), and an endogenous ligand of PPAR γ (15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15-deoxy- $\Delta^{12,14}$ -PGJ₂)) on K_{ATP}-channel activity in clonal hamster insulinoma cell line, HIT-T15 cells. As assessed by whole-cell patch clamp, fenofibrate, fenofibric acid, troglitazone, and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ reduced the K_{ATP}-channel currents, and inhibition continued after washout of these agents. The concentration-response curves of fenofibrate, fenofibric acid, troglitazone, and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ showed half maximal inhibition of K_{ATP}-channel currents (IC₅₀) at 3.26 $\mu\text{mol/l}$, 94 $\mu\text{mol/l}$, 2.1 $\mu\text{mol/l}$ and 7.3 $\mu\text{mol/l}$ respectively. Fenofibrate ($\geq 10^{-6}$ mol/l), 15-deoxy- $\Delta^{12,14}$ -PGJ₂ ($\geq 5 \times 10^{-5}$ mol/l) and troglitazone ($\geq 10^{-6}$ mol/l) inhibited [³H]-glibenclamide binding, but fenofibric acid did not. In addition, fenofibrate ($\geq 10^{-6}$ mol/l), fenofibric acid (10^{-4} mol/l), troglitazone (10^{-4} mol/l), and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ ($\geq 10^{-5}$ mol/l) increased insulin secretion from HIT-T15 when applied for 10 min. Our data suggest that PPAR α and γ ligands directly interact with the β -cell membrane and stimulate insulin secretion.

Introduction

Insulin secretion is stimulated by the closure of the ATP-sensitive potassium (K_{ATP}) channel of the β -cell membrane (Cook et al., 1984; Stergess et al., 1985; Rorsman and Trube, 1985; Ashcroft and Gribble, 1999). The K_{ATP} channel plays a major role in regulating the membrane potential of β -cells as it is able to respond to the metabolic state of the cell. The reduction in membrane K^+ permeability by closure of the K_{ATP} channel depolarizes the β -cell membrane. As a consequence of the change in membrane potential to more positive potentials, the voltage dependent Ca^{2+} channel (VDCC) opens to allow Ca^{2+} influx, and stimulates insulin release (Rorsman, 1997; Ashcroft and Trube, 1999). Sulfonylureas, such as tolbutamide and glibenclamide, stimulate insulin secretion by interacting directly with the K_{ATP} channel (Stergess et al., 1985; Edwards et al., 1993). K_{ATP} channels in pancreatic β -cells are composed of an inwardly rectifying Kir6.2 channel subunit, which forms the channel pore, and sulfonylurea receptor (SUR) 1 subunit, which acts as a regulatory subunit (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997; Ashcroft and Gribble, 1999). Sulfonylureas are known to bind directly to SUR 1 to inhibit the K_{ATP} channel (Stergess et al., 1985; Edwards et al., 1993; Ashcroft and Gribble, 1999).

On the other hand, intracellular nuclear receptors, peroxisome proliferator-activated receptors (PPARs), have evident effects on protein synthesis (Braissant et al., 1996). PPAR subtypes, α , γ and δ , show distinctive tissue distributions, and are associated with selective ligands (Braissant et al., 1996). Once activated by ligands, PPARs heterodimerize with retinoic X receptor and alter the transcription of target genes after binding to response elements, consisting of a direct repeat of the nuclear receptor DNA recognition motif spaced by one nucleotide. PPAR α is known to regulate fatty acid metabolism by controlling its oxidation, and PPAR γ is known to involve in glucose homeostasis and adipocyte proliferation (Lemberger et al., 1996; Corton et al., 2000). Fenofibrate is a PPAR α ligand and clinically used for the treatment of hyperlipidemia. Fenofibrate is rapidly converted into fenofibric acid in the liver and plasma. It is assumed that the fenofibric acid is the pharmacologically relevant form (Caldwell, 1989). On the other hand, troglitazone is a PPAR γ ligand, and 15-deoxy-

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$\Delta^{12,14}$ -PGJ₂ is known to be an endogenous ligand of PPAR γ (Kliwer et al., 1995)..

We have previously demonstrated that both troglitazone and pioglitazone, PPAR γ ligands, stimulate acute-phase insulin secretion with a rapid increase of cytoplasmic Ca²⁺, but inhibit chronic-phase insulin secretion in a Ca²⁺-dependent manner (Ohtani et al., 1996; 1998). Removing extracellular Ca²⁺ abolished acute-phase insulin secretion by troglitazone and pioglitazone. The effects of PPAR α agonists on insulin secretion remain unclear, but it is generally believed that these ligands do not influence insulin secretion when used clinically. However, in this present study, we have found that a PPAR α ligand (fenofibrate, fenofibric acid), a PPAR γ ligand (troglitazone) and an endogenous ligand of PPAR γ (15-deoxy- $\Delta^{12,14}$ -PGJ₂) inhibit the K_{ATP} channel directly and may induce insulin secretion. These results indicate the existence of a new pharmacological action of PPAR ligands, when used clinically.

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Material and Methods

Chemicals

Fenofibrate, fenofibric acid, and troglitazone were kindly provided by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan) and Sankyo Company, Ltd. (Tokyo, Japan). The concentration of fenofibrate and fenofibric acid used in these experiments were based on the circulating concentration of fenofibric acid (Caldwell, 1989). The concentration of troglitazone used in this experiment was chosen because it has been shown to be clinically relevant in studies of peripheral insulin resistance (Shibata et al., 1993). Fetal bovine serum was purchased from GIBCO (Grand island, NY, USA), F-12K medium in powder form was purchased from Flow Laboratories Inc. (Irvine, Scotland, UK). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

HIT-T15 cells were purchased from Flow Laboratories, Inc. (Irvine, Scotland, UK). The cells were cultured in F-12K medium containing 7mmol /l glucose and supplemented with 10% FBS, and incubated in a 95% O₂- 5% CO₂ incubator at 37°C.

Electrophysiological experiments

K_{ATP} currents were recorded in the whole-cell patch-clamp configuration using Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA). The standard extracellular solution contained 160 mmol /l NaCl, 5 mmol /l KCl, 1 mmol /l MgCl₂, 2 mmol /l CaCl₂, 10 mmol /l HEPES, 0.5 mmol /l Glucose. The pH of the extracellular solution was adjusted to 7.4 with NaOH. The pipette solution contained 150 mmol /l KCl, 10 mmol /l HEPES, 11mmol /l EGTA, 1 mmol /l MgCl₂ and 1 mmol /l CaCl₂. The pH of the pipette solution was adjusted to 7.2 with KOH. The resistance of patch

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pipettes when filled with the pipette solution ranged from 2 to 4 M Ω . Following gigaohm seal formation negative pressure was applied to the pipette to rupture the membrane and establish the whole-cell mode. The K_{ATP} currents were measured by repeatedly applying ramp pulses from -90 to -50 mV. All the experiments were completed within 10 min after establishing the whole-cell configuration. One concentration of fenofibrate, fenofibric acid, troglitazone or 15-deoxy- $\Delta^{12,14}$ -PGJ₂ was applied to each cell. The reversal potential of the HIT-T15 cell current was -60.8 mV under our experimental conditions. Electrophysiological experiments were performed at room temperature (22-25 °C).

Binding experiments

HIT-T15 cells were resuspended at a density of 1.5×10^7 cells /ml in glucose-free F-12K medium. Competitive inhibition assays were performed with 1.0 nmol /l [³H]-glibenclamide, and various concentrations of fenofibrate, fenofibric acid, troglitazone, and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ were incubated for 1 hour at room temperature (Masuda et al., 1995). Incubation was terminated by rapid filtration under a vacuum with a cell harvester and Multiscreen FC glass fiber filters (Mutiscreen separation system, Millipore, Bedford, Massachusetts, USA), and cells were washed three times with F-12K medium. The radioactivity of the filters was measured using a liquid scintillation counter (Top Count, Packard) after the addition of 10 ml scintillation cocktail (Aquasol-2, New England Nuclear, Newton, Massachusetts, USA). Binding inhibition was expressed as a percentage of [³H]-glibenclamide specific binding.

Insulin secretion study

For insulin secretion studies, cells were plated on 24-multiwell plates (1×10^5 cells per well). On the day of the experiment, the culture medium was completely aspirated and replaced with fresh medium containing fenofibrate, fenofibric acid or 15-deoxy- $\Delta^{12,14}$ -PGJ₂.

The direct effect of fenofibrate (10^{-7} to 10^{-4} mol /l), fenofibric acid (10^{-7} to 10^{-4}

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mol /l), troglitazone (10^{-7} to 10^{-4} mol /l) and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (10^{-7} to 10^{-4} mol /l) on acute-phase insulin secretion were examined by the static incubation of HIT-T15 cells. Fenofibrate, fenofibric acid and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ were dissolved in dimethyl sulfoxide (DMSO) and methyl acetate with a final concentration of DMSO or methyl acetate below 0.05% in the culture medium (F-12K medium without FBS), and the same concentration was used with all groups to avoid the influence of increased osmolarity on insulin secretion.

After reaching confluence, cells were washed twice with fresh F-12K medium and incubated for 10 min in 1ml of experimental media. The medium was then aspirated and cells were centrifuged at 14000 rpm for 10 min. The supernatant was removed and frozen until radioimmune assay of insulin concentration by commercial RIA kits (Phadeceph Insulin, Pharmacia Japan, Tokyo, Japan).

Data analysis

The concentration-dependence of fenofibrate and fenofibric acid on K_{ATP}-channels were determined by fitting the following equation:

$$\frac{I}{I_c} = \frac{1}{1 + ([X]/IC_{50})^h} \quad [1]$$

where I is the current at -50mV in the presence of the ligands and I_c is the current at -50mV measured in the control solution. Both I and I_c were obtained by subtracting the measured current amplitudes during an exposure to $100 \mu\text{mol/l}$ tolbutamide. $[X]$ is the concentration of fenofibrate, fenofibric acid, troglitazone or 15-deoxy- $\Delta^{12,14}$ -PGJ₂, IC_{50} is the concentration at which inhibition is half maximal, and h is the Hill coefficient (slope factor).

The relationships between drug concentrations and specific binding were fitted by the following equation:

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$$y = \frac{a-b}{1+(K_i/[X])^h} + b \quad [2]$$

where y is the specific binding; K_i is the concentration at which binding is half maximal; $[X]$ is the concentration of fenofibrate, fenofibric acid, troglitazone or 15-deoxy- $\Delta^{12,14}$ -PGJ₂, h is the Hill coefficient, a is the upper plateau (maximum value for specific binding), and b is the lower plateau.

All data represent the mean \pm SE. The statistical analysis of the means was performed by analysis of variance (ANOVA), followed by Duncan's multiple range test for the individual comparisons of the means.

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Results

Effect of PPAR α ligand (fenofibrate) on K_{ATP} channel activity.

Figure 1A (1) shows a HIT-T15 whole-cell current in response to voltage ramps from -90 to -50 mV in standard extracellular solution. As shown in the control current, the current amplitudes evoked by ramps were stable over 10min under our experimental conditions. Subsequent application of tolbutamide (10^{-4} mol /l) reduced the conductance by 94.3 ± 5.2 % (n=36), suggesting that the currents principally reflect the activity of K_{ATP} channels. Application of an extracellular solution containing 22.2mmol /l glucose did not affect K_{ATP}-channel current indicating that the intracellular complex including the glycolysis system is replaced by the pipette solution in the whole-cell mode. Therefore, factors such as glucose metabolism do not interfere with the K_{ATP} channel under our experimental condition.

As shown in Figure 1A (2), the K_{ATP}-channel current activity was inhibited slowly after the application of fenofibrate (approximately 3~5 min after the application to reach maximal inhibition), and the inhibition continued after the washout of fenofibrate. We have also confirmed the depolarization of the β -cell membrane potential after applying fenofibrate in current-clamp mode (data not shown). Figure 1C shows the relationship between fenofibrate concentrations and relative current of K_{ATP}-channel currents. The inhibitory effect of the K_{ATP}-channel current was maximum at the concentration of 1×10^{-4} mol /l of fenofibrate. The IC₅₀ of inhibiting the K_{ATP} channel current by fenofibrate was 3.3 ± 0.5 μ mol /l (n=8) and the Hill coefficient was 1.1 ± 0.2 .

Effect of PPAR α ligand (fenofibric acid) on K_{ATP} channel activity.

Figure 2A (1) shows a HIT-T15 cell K_{ATP}-channel current in response to voltage ramps from -90 to -50 mV in standard extracellular solution. Fenofibric acid, the active form of fenofibrate, irreversibly inhibited the K_{ATP} current and maximal inhibition was reached 3~5 min after exposure (Fig. 2A(2)). The concentration-dependent relationship

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of K_{ATP} -channel current for fenofibric acid is shown in Fig. 2C. The IC_{50} for fenofibric acid on K_{ATP} channels was $94 \pm 0.5 \mu\text{mol/l}$ ($n=8$) and the Hill coefficient was 2.1 ± 0.26 .

Effect of PPAR γ ligand (troglitazone) on K_{ATP} -channel currents.

Figure 3A (1) shows a control K_{ATP} -channel current in response to voltage ramp pulses from -90 to -50 mV in standard extracellular solution. Troglitazone also irreversibly inhibited K_{ATP} -channel current and again maximal inhibition was obtained 3~5 min after exposure of the cells to the drug. (Fig. 3A(2)). The concentration-effect relationship troglitazone on the K_{ATP} currents is shown in Fig. 3C. The IC_{50} for troglitazone was $2.1 \pm 0.4 \mu\text{mol/l}$ ($n=8$) and the Hill coefficient was 1.9 ± 0.32 .

Effect of endogenous ligand of PPAR γ (15-deoxy- $\Delta^{12,14}$ -PGJ $_2$) on K_{ATP} -channel currents.

Figure 4A (1) shows a representative K_{ATP} -channel current recorded from an HIT-T15 cell in standard extracellular solution. 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ had effects similar to the other PPAR agonists since the K_{ATP} current became irreversibly inhibited and maximal inhibition was reached approximately 3~5 min after the exposure (Fig. 4A(2)). The concentration-effect curve for 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ inhibition of K_{ATP} currents is shown in Fig. 4C. The IC_{50} for 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ inhibition was $7.3 \pm 0.96 \mu\text{mol/l}$ ($n=8$) and the Hill coefficient was 1.0 ± 0.13 .

Inhibition binding assay with PPAR α ligands.

To examine the possibility that PPAR α ligands may bind to the same site as glibenclamide, we performed an inhibition binding assay with 1.0 nmol/l [^3H]-glibenclamide and various concentrations of fenofibrate or fenofibric acid.

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The equilibrium dissociation constant (K_D) of [3 H]-glibenclamide was 0.18 nmol /l in HIT-T15 cells, consistent with the value of 0.22 nmol /l reported by Schwanstecher et al (1992).

As shown in Figure 5A, fenofibrate inhibited the binding of [3 H]-glibenclamide at concentrations higher than unlabelled glibenclamide. Fenofibrate inhibited the binding of [3 H]-glibenclamide at concentrations higher than 1×10^{-7} mol /l (74.3 ± 3.2 % at 1×10^{-6} mol /l, 63.5 ± 1.8 % at 1×10^{-5} mol /l and 56.3 ± 4.3 % at 1×10^{-4} mol /l, n=3), whilst fenofibric acid failed to inhibit the binding of [3 H]-glibenclamide at the concentrations tested (Fig. 5B). The results suggest that at these concentrations fenofibrate binds to the same site as glibenclamide, but fenofibric acid may occupy a separate site.

Inhibition binding assay with PPAR γ ligands.

Similarly, PPAR γ ligands, 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ and troglitazone, inhibited [3 H]-glibenclamide binding at concentrations higher than unlabelled glibenclamide. Inhibition by 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ to HIT-T15 cells was observed at concentrations higher than 1×10^{-6} mol /l (60.2 ± 0.1 % at 5×10^{-6} mol /l, 50.0 ± 0.1 % at 1×10^{-5} mol /l and 40.0 ± 0.01 % at 5×10^{-5} mol /l, n=3) (Fig. 6A). Troglitazone inhibited [3 H]-glibenclamide binding at concentrations higher than 1×10^{-6} mol /l (79.9 ± 3 % at 1×10^{-5} mol /l and 52.7 ± 6.5 % at 1×10^{-4} mol /l, n=3) (Fig. 6B).

These results suggest that both troglitazone and 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ bind to the same site as glibenclamide.

Effect of PPAR α ligand (fenofibrate, fenofibric acid) and PPAR γ ligand (15-deoxy- $\Delta^{12,14}$ -PGJ $_2$) on acute-phase insulin secretion.

Figure 7 shows the acute-phase insulin secretion after static incubation with fenofibrate (10^{-7} ~ 10^{-4} mol /l) (Fig. 7A), fenofibric acid (10^{-7} ~ 10^{-4} mol /l) (Fig. 7B), troglitazone

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($10^{-7} \sim 10^{-4}$ mol /l) (Fig 7C) and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ ($10^{-7} \sim 10^{-4}$ mol /l) (Fig. 7D) for 10 min in F-12K medium containing 7 mmol /l glucose.

Acute-phase insulin secretion in the absence of fenofibrate was 2.3 ± 0.4 ng /ml (n=8). Fenofibrate significantly stimulated insulin secretion at concentrations of 10^{-6} mol /l (3.6 ± 0.3 ng /ml, n=8), 10^{-5} mol /l (4.0 ± 0.6 ng /ml, n=8) and 10^{-4} mol /l (4.0 ± 0.3 ng /ml, n=8) (Fig. 7A).

The effect of fenofibric acid, the active form of fenofibrate, on insulin secretion was also examined. Under drug-free conditions, insulin secretion was 2.0 ± 0.1 ng /ml (n=8). Fenofibric acid only gave a statistically significant increase in insulin secretion at a concentration of 10^{-4} mol /l (2.6 ± 0.1 ng /ml, n=8) (Fig. 7B).

These stimulatory effects of fenofibrate and fenofibric acid on insulin secretion were also observed at a glucose concentration of 3 mmol /l, where the amount of acute-phase insulin secretion in control medium was 1.2 ± 0.3 ng /ml (n=8). Fenofibrate significantly increased the amount of insulin secreted at concentrations of 10^{-5} mol /l (2.1 ± 0.2 ng /ml, n=8, $p < 0.05$ vs. control) and 10^{-4} mol /l (3.3 ± 0.7 ng /ml, n=8, $p < 0.01$ vs. control). In the fenofibric acid experiment, secretion under control conditions was also low (1.0 ± 0.8 ng /ml, n=8), but fenofibric acid only significantly stimulated the insulin secretion at a concentration of 10^{-4} mol /l (2.1 ± 0.7 ng /ml, n=8, $p < 0.05$ vs. control).

Exposures to the PPAR γ ligand, troglitazone, for 10 min also enhanced insulin secretion. Under drug-free conditions insulin secretion was 1.5 ± 0.3 ng/ml (n=8), whilst in the presence of 10^{-4} mol/l Troglitazone, insulin secretion was 2.6 ± 0.1 ng/ml (n=8, Fig 7C).

The effects of the endogenous PPAR γ ligand 15-deoxy- $\Delta^{12,14}$ -PGJ₂ on insulin secretion was also tested. Under control conditions, insulin secretion was 2.9 ± 0.3 ng /ml, and 10^{-5} mol /l 15-Deoxy- $\Delta^{12,14}$ -PGJ₂ increased secretion to 4.9 ± 0.6 ng /ml and 10^{-4} mol /l 15-Deoxy- $\Delta^{12,14}$ -PGJ₂ to 6.4 ± 0.5 ng /ml (Fig. 7D).

Discussion

The present studies demonstrated that both PPAR α ligands (fenofibrate, fenofibric acid) and PPAR γ ligands (troglitazone, 15-deoxy- $\Delta^{12,14}$ -PGJ₂) inhibit K_{ATP} channel and induce acute-phase insulin secretion. Fenofibrate, 15-deoxy- $\Delta^{12,14}$ -PGJ₂ and troglitazone inhibited the binding of [³H]-glibenclamide. These results indicated that PPAR α and γ ligands have a direct effect on the β -cell membrane, which may be independent of the PPAR pathway in the cytoplasm.

Glibenclamide and tolbutamide reportedly show different patterns in inhibiting K_{ATP}-channel current. Glibenclamide is known to inhibit pancreatic β -cell K_{ATP}-channel current irreversibly (Sturgess et al., 1988; Zunkler et al., 1988; 1989; Zunkler et al., 1989; Gribble et al., 1998; Ashcroft and Gribble, 1999). In contrast, tolbutamide inhibits pancreatic β -cell K_{ATP}-channel current in a reversible manner (Stergess et al., 1988; Gribble et al., 1998; Ashcroft and Gribble, 1999; Babenko et al., 1999). In the present study, we have shown that fenofibrate, fenofibric acid, troglitazone, and 15-deoxy- $\Delta^{12,14}$ -PGJ₂, all inhibit the K_{ATP}-channel current and the inhibition continues after the removal of the ligands. This indicates that fenofibrate, fenofibric acid, troglitazone and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ may inhibit the K_{ATP} channel in a similar manner to glibenclamide. In addition, the fact that fenofibrate, troglitazone and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ inhibit the binding of [³H]-glibenclamide suggests that these PPAR ligands may interact directly with SUR. Therefore, we speculate that fenofibrate, troglitazone, and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ stimulate insulin secretion by binding to SUR and inhibiting the K_{ATP} channel, similar to sulfonylureas. Although the binding site on SUR of these ligands may not necessarily be the same as that for glibenclamide, we propose that they may bind to a site on SUR1 or related molecules and consequently close the channel. However, the fact that these PPAR ligands inhibit the binding of [³H]-glibenclamide, raise the possibility that these PPAR ligands may weaken the effect of glibenclamide, when administered together in clinical use.

In this study, we have shown that fenofibrate, but not fenofibric acid inhibited the binding of [³H]-glibenclamide. We speculate that the structural difference between fenofibrate and fenofibric acid may contribute to these different effects (Fig. 8).

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Fenofibrate is rapidly converted into the carboxic acid form, fenofibric acid, by esterases in the liver and plasma (Caldwell, 1989). A chemical moiety lacking in the structure of fenofibric acid compared to fenofibrate could be important in the binding inhibition of [³H]-glibenclamide. The fact that fenofibric acid also has an inhibitory effect on K_{ATP} channels suggests that fenofibric acid has an inhibitory effect on K_{ATP} channels by a different mechanism from that of fenofibrate, 15-deoxy- $\Delta^{12,14}$ -PGJ₂ and troglitazone.

The finding that fenofibrate stimulated insulin secretion and inhibited K_{ATP}-channel activity may not be consistent with the fact that fenofibrate does not perturb glucose homeostasis. To date, there are no reports of hypoglycemia from patients administering fenofibrate as a treatment for hyperlipidemia. Fenofibrate is rapidly converted into fenofibric acid in the liver and plasma, and blood fenofibrate declines to undetectable levels (Caldwell, 1989). It has been assumed that the pharmacologically relevant form of fenofibrate is the acid form. In this study, we have shown that fenofibric acid stimulated insulin secretion only at a concentration of 10⁻⁴ mol /l and that the magnitude of insulin secretion stimulated by fenofibric acid was lower than fenofibrate. The IC₅₀ for inhibition of K_{ATP} channels by fenofibric acid was 9.4 x 10⁻⁵ mol /l, a value much higher than actual circulating concentration of fenofibric acid (Caldwell, 1989). Therefore, fenofibrate may hardly influence actual insulin secretion in clinical use.

Holness et al (2003) have reported that 24 h application of PPAR α agonist, WY 14,643 opposed insulin secretion elicited by high fat feedings in rats. Tordjman et al (2002) also reported that over expression of PPAR α in INS-1 cells, an insulinoma cell line, decreased basal and glucose-stimulated insulin secretion. These two reports clearly demonstrated that chronically activating PPAR α will suppress insulin secretion. We also observed that the insulin secretion stimulated by fenofibrate or fenofibric acid does not persist when these compounds were applied for more than 2 h in HIT-T15 cells (data not shown). These chronic effects on β -cells of PPAR α ligands may be produced by the mechanism of PPAR α dependent pathway and this pathway may not be involved in the acute effects that we demonstrate in this investigation.

It is well known that there are PPAR-independent effects exerted by PPAR γ ligands such as 15-deoxy- $\Delta^{12,14}$ -PGJ₂ and troglitazone. These include effects caused by

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on microvascular endothelial cells and skeletal muscle (Brunmair et al., 2001; Jozkowicz et al., 2001). PPAR γ -independent effects of 15-deoxy- $\Delta^{12,14}$ -PGJ₂ and ciglitazone are involved in the generation of interleukin-8 by the human microvascular endothelial cell line (Jozkowicz et al., 2001). Troglitazone may have a PPAR γ -independent effect on skeletal muscle fuel metabolism (Brunmair et al., 2001). It has been shown that 15-deoxy- $\Delta^{12,14}$ -PGJ₂ has a direct effect on the I κ B kinase complex and NF- κ B activation in HeLa cells, which do not express PPAR γ (Rossi et al., 2000). It was also reported that 15-deoxy- $\Delta^{12,14}$ -PGJ₂ impairs cytokine signaling by inhibiting I κ B, stimulating IFN- γ , and nitric oxide generation independently of PPAR γ in pancreatic β -cells (Weber, 2003). These cytokines or generation of nitric oxide may also influence insulin secretion from pancreatic β -cells. The effects of PPAR ligands in our study are clearly different from the PPAR-independent effects reported by Weber et al (2003). Our study revealed that PPAR ligands stimulate insulin secretion and inhibit K_{ATP}-channel activity in the whole-cell configuration in a very short time (10 min). Cytokine signalings or nitric oxide generation cannot stimulate insulin secretion within 10 min under our experimental conditions, because intracellular complexes including cytokines are replaced by a pipette solution during the whole-cell mode. In addition, Ogawa et al (1999) reported that application of a low concentration of troglitazone (3 μ mol/l) does not induce insulin secretion in HIT-T15 cells. Our result showed that troglitazone inhibited K_{ATP} channel and stimulated acute-phase insulin secretion in a comparatively high concentration. Therefore, we speculate that troglitazone may inhibit K_{ATP} channel and induce insulin secretion only at the higher concentration. Our study strongly indicates the existence of the PPAR-independent pathway of its ligands, which directly inhibit the K_{ATP} channel in the β -cell membrane. However, the concentration relation between PPAR ligands and K_{ATP} channel inhibition does not completely correlate with the concentration relation between PPAR ligands and amount of acute-phase insulin secretion. Recently, there have been reports indicating that voltage-dependent K⁺ (K_v) channels and ATP-dependent Ca²⁺ pumping, in addition to the K_{ATP} channel, control the β -cell membrane potential and the threshold for insulin secretion (Kanno et al., 2002; MacDonald and Wheeler, 2003). The lack of complete correlation in concentration between K_{ATP} channel inhibition and insulin secretion in our study may

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be explained by these factors other than K_{ATP} channel which control the membrane potential. Further studies are required to determine the precise mechanism of PPAR ligands on acute-phase insulin secretion.

The present finding that troglitazone inhibited K_{ATP} channels is in contrast to the results reported by Masuda et al (1995). They did not observe the inhibitory effect of troglitazone on K_{ATP} -channel activity despite the stimulation of insulin secretion. This may be caused because troglitazone was applied at a concentration lower (10^{-6} mol /l) than we used and thus difficult to detect the inhibitory effect on the K_{ATP} channels.

Recently, Heron et al (1998) have reported that α -endosulfine may act as an endogenous regulator of β -cell K_{ATP} channel activity and insulin secretion. α -endosulfine is a peptide that was purified from bovine brain in the quest for an endogenous ligand for the SUR in brain (Virsolvy-Vergine 1988). In the present study, we have shown that the endogenous ligand for PPAR γ , 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$, may bind to SUR and inhibit K_{ATP} channels. Prostaglandins (PGs) are known to play an important role in biological processes and PGD $_2$ is a major cyclooxygenase product in a variety of tissues having a marked biological effect (Giles and Leff, 1988). It has been reported that PGD $_2$ undergoes dehydration to the J $_2$ series in both *in vitro* and *in vivo* experiments (Fitzpatrick et al., 1983; Kikawa et al., 1984). One of the J $_2$ series, 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$, is known to be the endogenous ligand of PPAR γ (Kilwer et al., 1995). The present study adds the possibility that 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ may also act as an endogenous regulator of β -cell K_{ATP} channel activity and insulin secretion by binding directly to SUR1.

K_{ATP} channels are known to exist not only in pancreatic β -cells but also in various tissues such as neurons, cardiac muscle, skeletal muscle and smooth muscle. In these tissues, different types of SURs serve as the regulatory subunits of K_{ATP} channels in association with pore-forming Kir6.x subunits: SUR1 in pancreatic β -cells and neurons, SUR2A in cardiac and skeletal muscles and SUR2B in smooth muscle (Sakura et al., 1995; Chutkow et al., 1996; Ishimoto et al., 1996; Inagaki et al., 1995; 1996; 1997; Yamada et al., 1997; Karschin et al., 1998). It is intriguing to consider if PPAR ligands may have similar effects on [3 H]-glibenclamide-binding to the SUR isoforms other than SUR1.

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Figure Legends

Fig. 1. Effect of fenofibrate on K_{ATP} -channel current from HIT-T15 cells. K_{ATP} -channel current was measured in response to voltage ramps from -90 mV to -50 mV using the standard whole cell patch clamp configuration. **A:** Representative K_{ATP} -channel current under our experimental conditions (1), effect of 10^{-4} mol /l fenofibrate (2) on K_{ATP} -channel current. The line above the current pulses represents the application time of fenofibrate. A section of control current trace in expanded time scale (3). The dotted line indicates the zero-current level. **B:** Concentration-response curve for inhibition of K_{ATP} -channel current by fenofibrate. Relationship between fenofibrate concentration and the macroscopic K_{ATP} current, expressed as a fraction of its amplitude in the absence of the drug (I/I_C). The currents fitted Eq.(1): $IC_{50} = 3.26 \mu \text{ mol /l}$, $h=1.14$. Values represent means \pm standard errors. Asterisks * indicate $p < 0.01$, + indicate $p < 0.05$ vs. control. $n=8$ in each group.

Fig. 2. Effect of fenofibric acid on K_{ATP} -channel current from the HIT-T15 cells. K_{ATP} -channel current was measured in response to the voltage ramps from -90 mV to -50 mV in the standard whole-cell configuration. **A:** Example of the K_{ATP} -channel current under our experimental conditions (1), effect of 10^{-4} mol /l fenofibric acid (2) on K_{ATP} -channel current. The line above the current pulses represents the application time of fenofibric acid. A sample of control current trace in an expanded time scale (3). The dotted line indicates the zero-current level. **B:** Concentration-response curve for inhibition of K_{ATP} -channel current by fenofibric acid. Relationship between the fenofibric acid concentration and the macroscopic K_{ATP} current, expressed as a fraction of its amplitude in the absence of the drug (I/I_C). The currents fitted Eq.(1): $IC_{50} = 94 \mu \text{ mol /l}$, $h=2.1$. Values represent means \pm standard errors. Asterisk * indicate $p < 0.01$ vs. control. $n=8$ in each group.

Fig. 3. Effect of troglitazone on K_{ATP} -channel current from the HIT-T15 cells. K_{ATP} -channel current was measured in response to voltage ramps from -90 mV to -50 mV in

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the standard whole-cell configuration. **A:** Example of the K_{ATP} -channel current under our experimental conditions (1), effect of 10^{-4} mol /l troglitazone (2) on K_{ATP} -channel current. The line above the current pulses represents the application time of troglitazone. A sample of control current trace in an expanded time scale (3). The dotted line indicates the zero-current level. **B:** Concentration-response curve for inhibition of K_{ATP} -channel current by troglitazone. Relationship between the troglitazone concentration and the macroscopic K_{ATP} current, expressed as a fraction of its amplitude in the absence of the drug (I/I_C). The currents fitted Eq.(1): $IC_{50}= 2.1 \mu$ mol /l, $h=1.9$. Values represent means \pm standard errors. Asterisk * indicate $p<0.01$ vs. control. $n=8$ in each group.

Fig. 4. Effect of 15-deoxy- Δ^{1214} -PGJ₂ on K_{ATP} -channel current from the HIT-T15 cells. K_{ATP} -channel current was measured in response to voltage ramps from -90 mV to -50 mV in the standard whole-cell configuration. **A:** Example of the K_{ATP} -channel current under our experimental conditions (1), effect of 4×10^{-5} mol/l 15-deoxy- Δ^{1214} -PGJ₂ (2) on K_{ATP} -channel current. The line above the current pulses represents the application time of fenofibric acid. A sample of control current trace in an expanded time scale (3). The dotted line indicates the zero-current level. **B:** Concentration-response curve for inhibition of K_{ATP} -channel current by 15-deoxy- Δ^{1214} -PGJ₂. Relationship between the 15-deoxy- Δ^{1214} -PGJ₂ concentration and the macroscopic K_{ATP} current, expressed as a fraction of its amplitude in the absence of the drug (I/I_C). The currents fitted Eq.(1): $IC_{50}= 7.3 \mu$ mol/l, $h=1.0$. Values represent means \pm standard errors. Asterisk * indicate $p<0.01$ vs. control. $n=8$ in each group.

Fig. 5. Inhibition of the specific [³H]-glibenclamide binding to HIT-T15 cells by increasing concentrations of unlabelled fenofibrate (A) and fenofibric acid (B). Fenofibrate inhibited the binding of [³H]-glibenclamide at higher concentrations than the unlabelled glibenclamide whilst fenofibric acid had no effect. Values represent the means \pm standard error. Asterisks * indicates $p<0.01$ vs. control, + indicates $p<0.05$ vs. control. $n=3$ in each group.

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Fig. 6. Inhibition of specific [³H]-glibenclamide binding to HIT-T15 cells by increasing concentrations of unlabelled 15-deoxy- Δ^{1214} -PGJ₂ (**A**) and troglitazone (**B**). 15-deoxy- Δ^{1214} -PGJ₂ and troglitazone inhibited the binding of [³H]-glibenclamide at higher concentrations than the unlabelled-glibenclamide. Values represent the means \pm standard error. Asterisks * indicates p<0.01 vs. control, + indicates p<0.05 vs. control. n=3 in each group.

Fig. 7. Effects of 10 min HIT-T15 cell exposure to fenofibrate (**A**), fenofibric acid (**B**), troglitazone (**C**) and 15-deoxy- Δ^{1214} -PGJ₂ (**D**) on acute-phase insulin secretion. Insulin secretion was stimulated at concentrations over 10⁻⁶ mol /l in fenofibrate, at a concentration of 10⁻⁴ mol /l in fenofibric acid, at a concentration of 10⁻⁴ mol /l in troglitazone and at over 10⁻⁵ mol /l in 15-deoxy- Δ^{1214} -PGJ₂. The glucose concentration in the medium used in this study was 7mmol /l. Values represent means \pm standard errors. Asterisks * indicate p<0.01 vs. control, + indicate p<0.05 vs. control. n=8 in each group.

Fig. 8. Structure of fenofibrate and fenofibric acid.

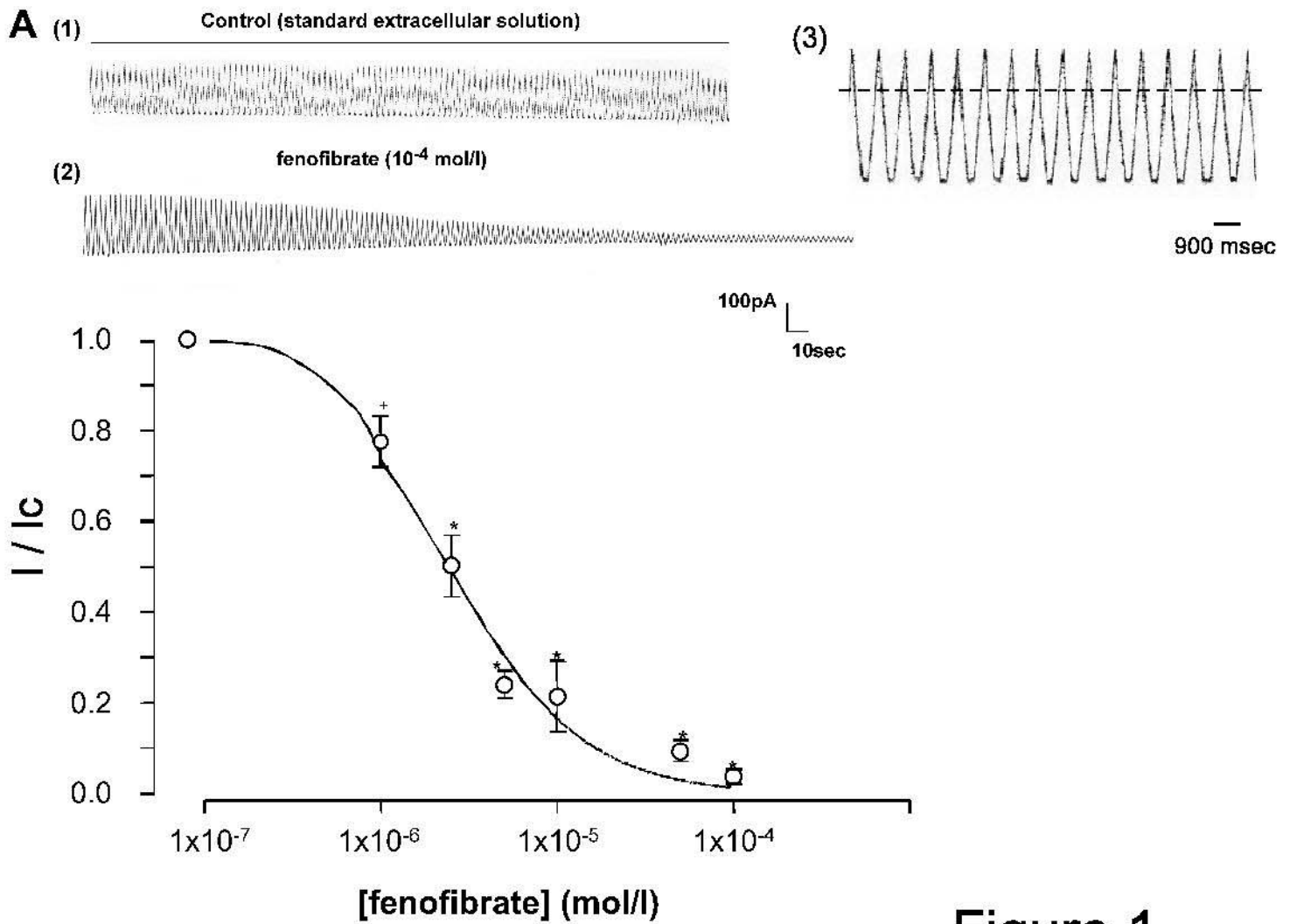


Figure 1

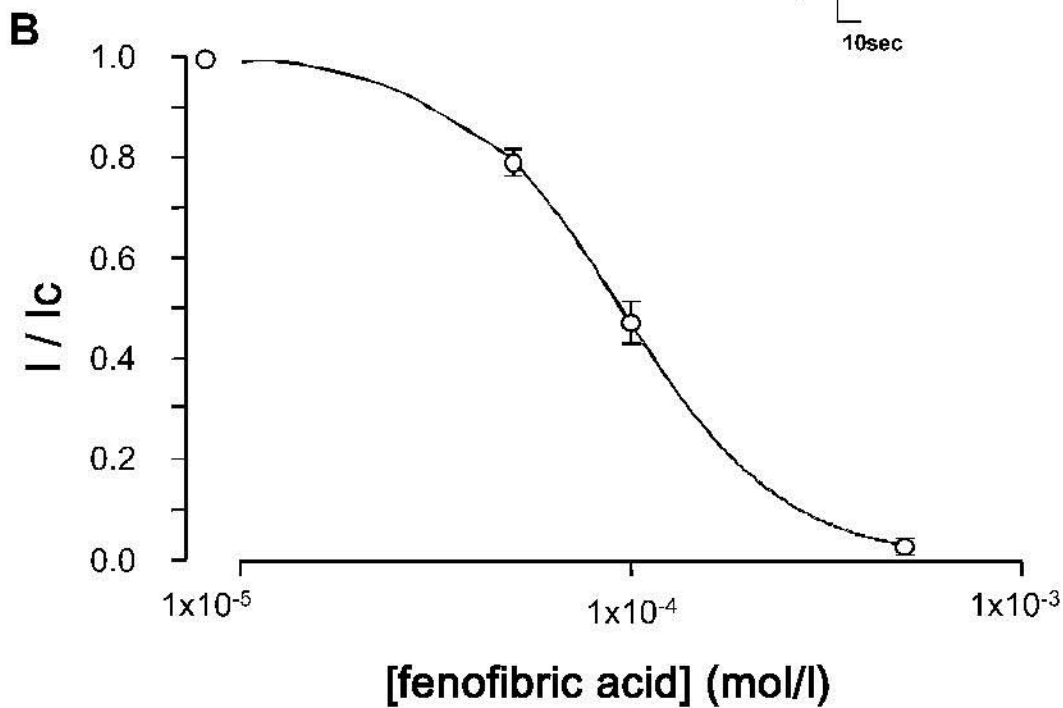
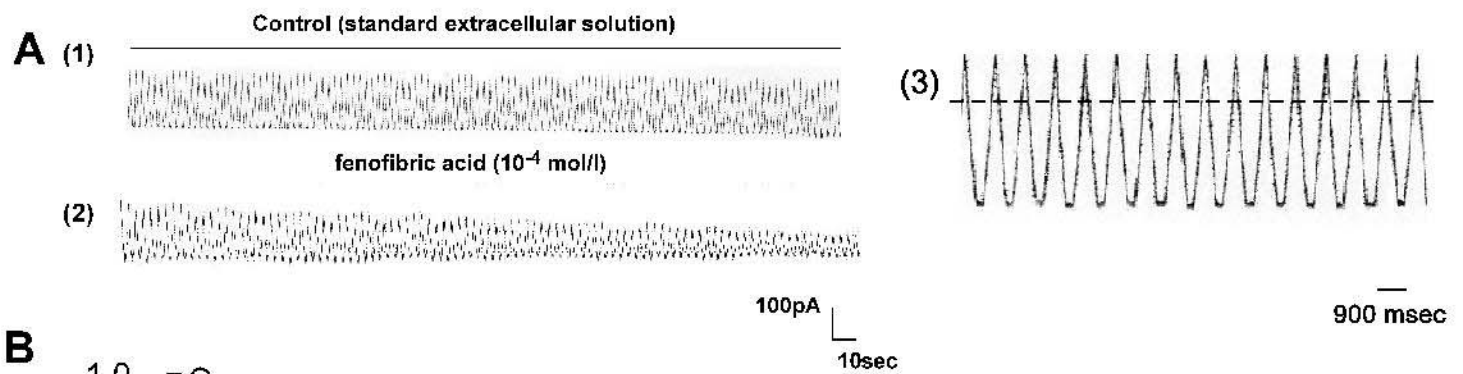


Figure 2

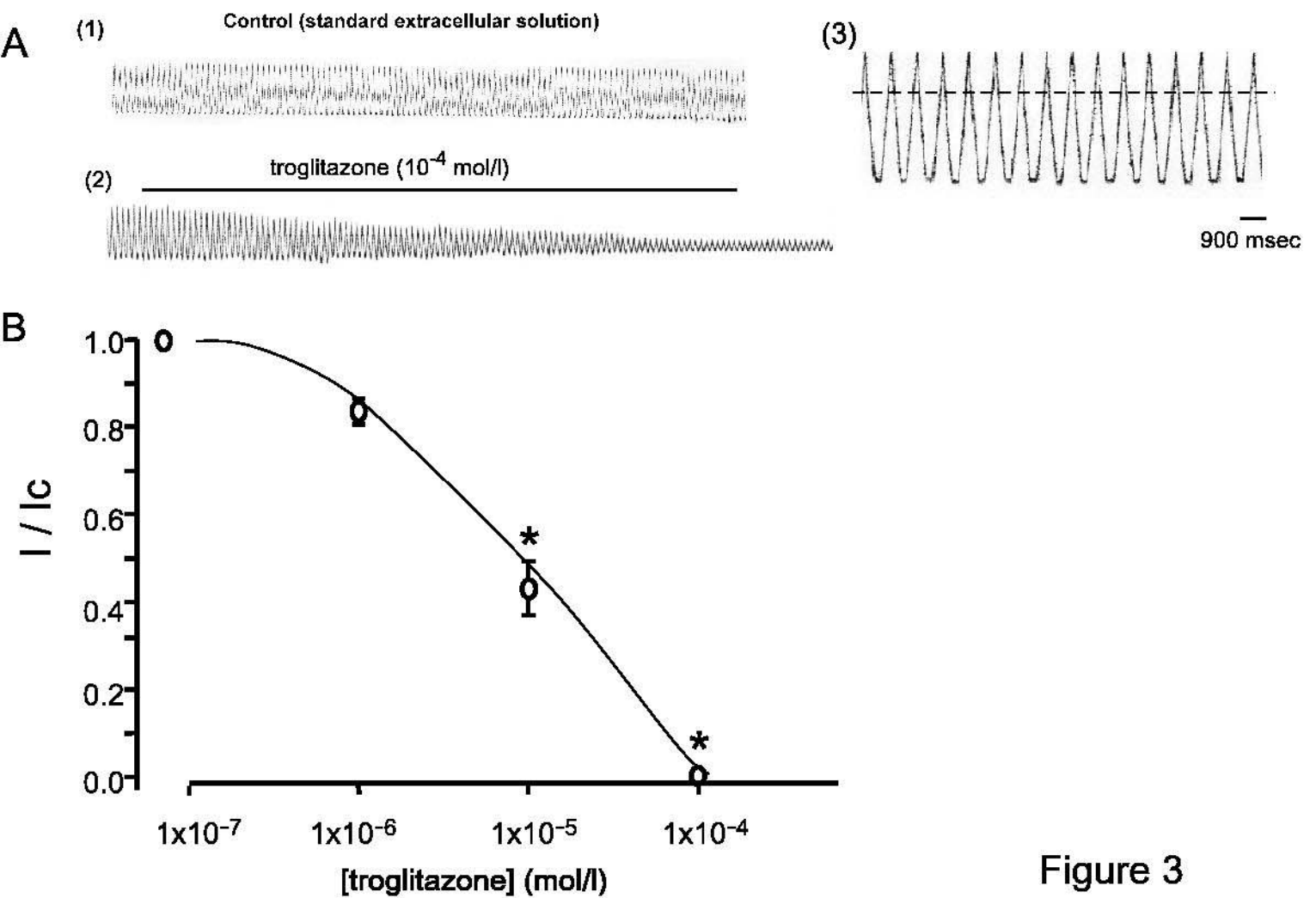


Figure 3

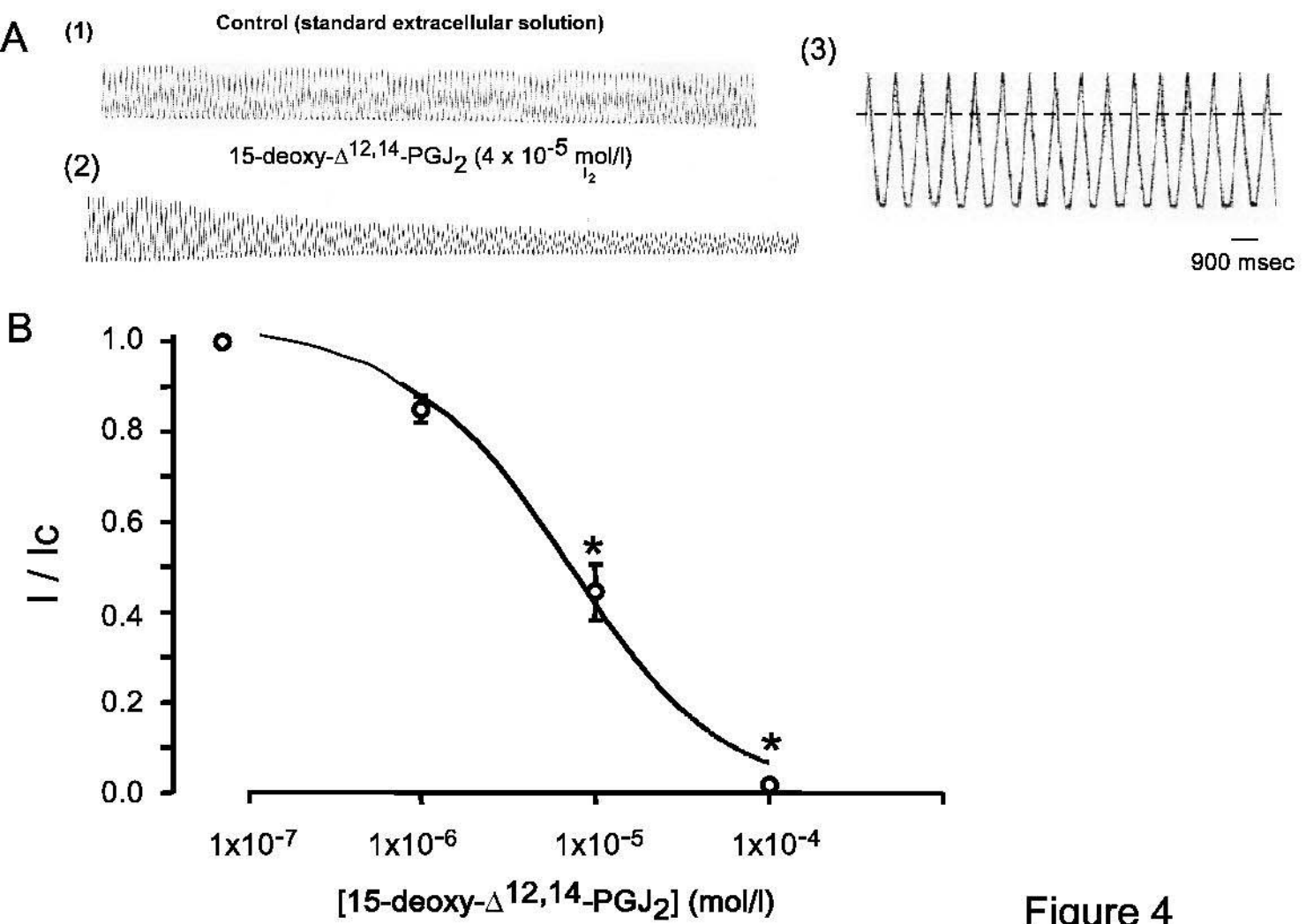


Figure 4

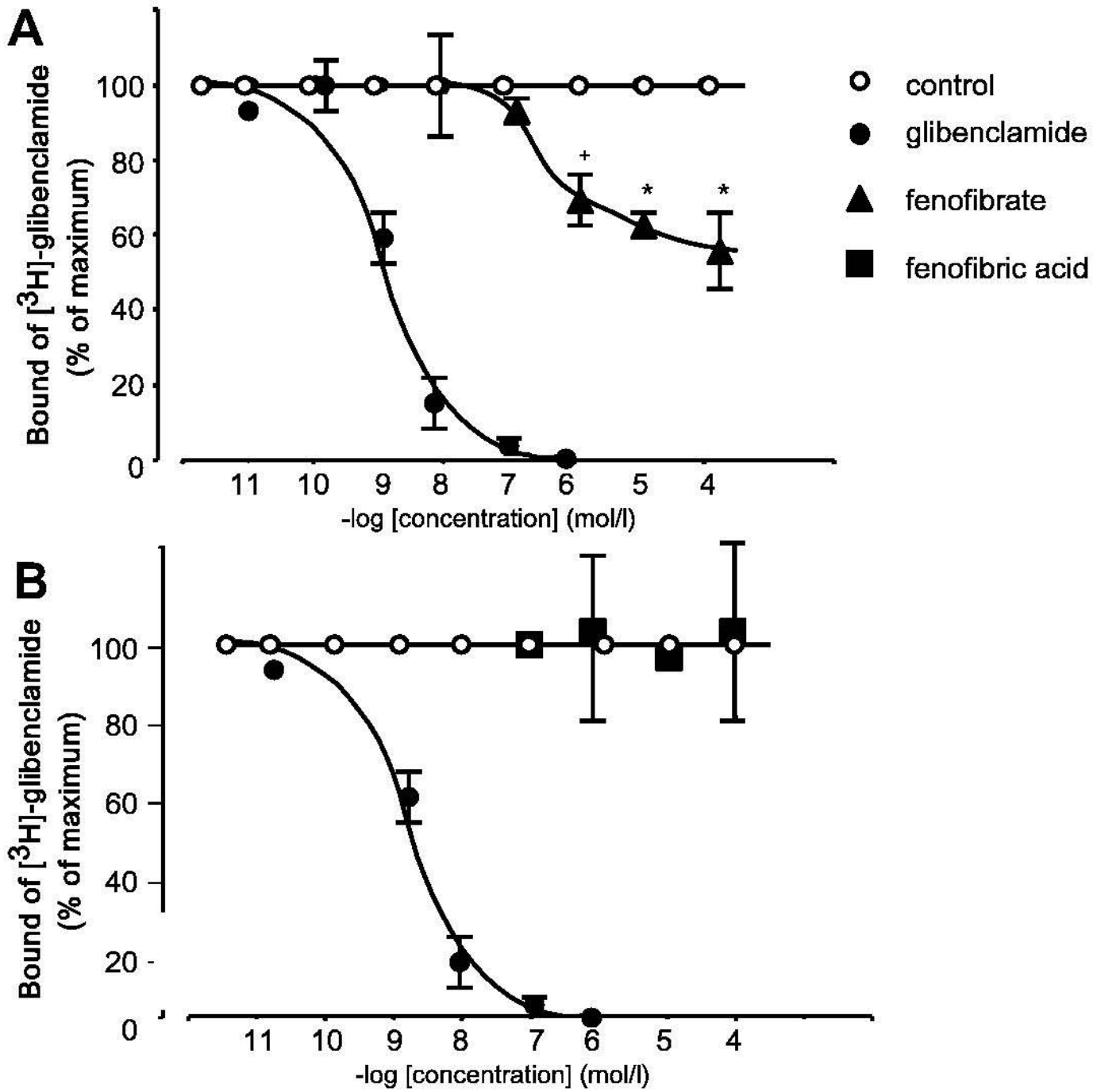


Figure 5

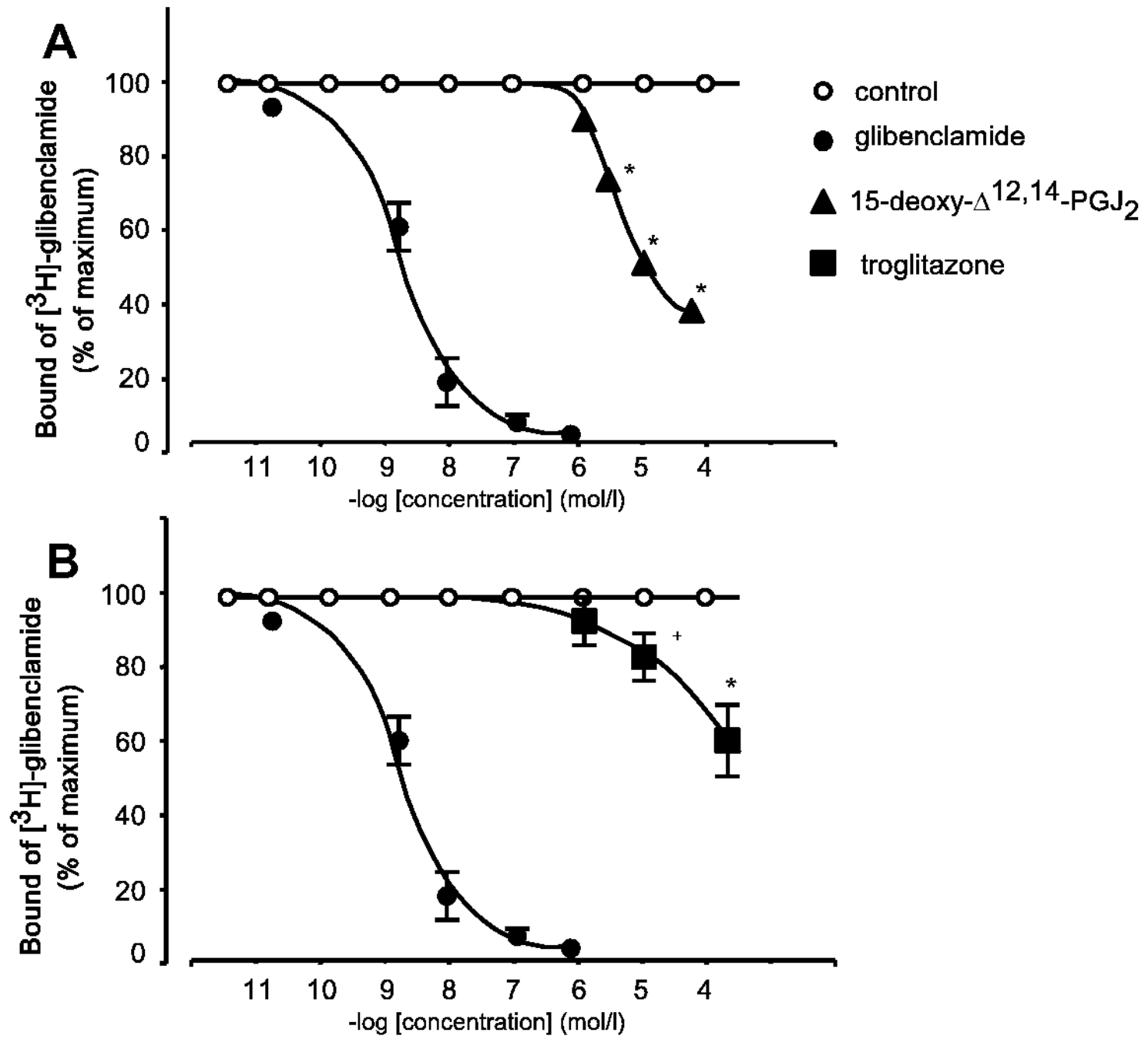


Figure 6

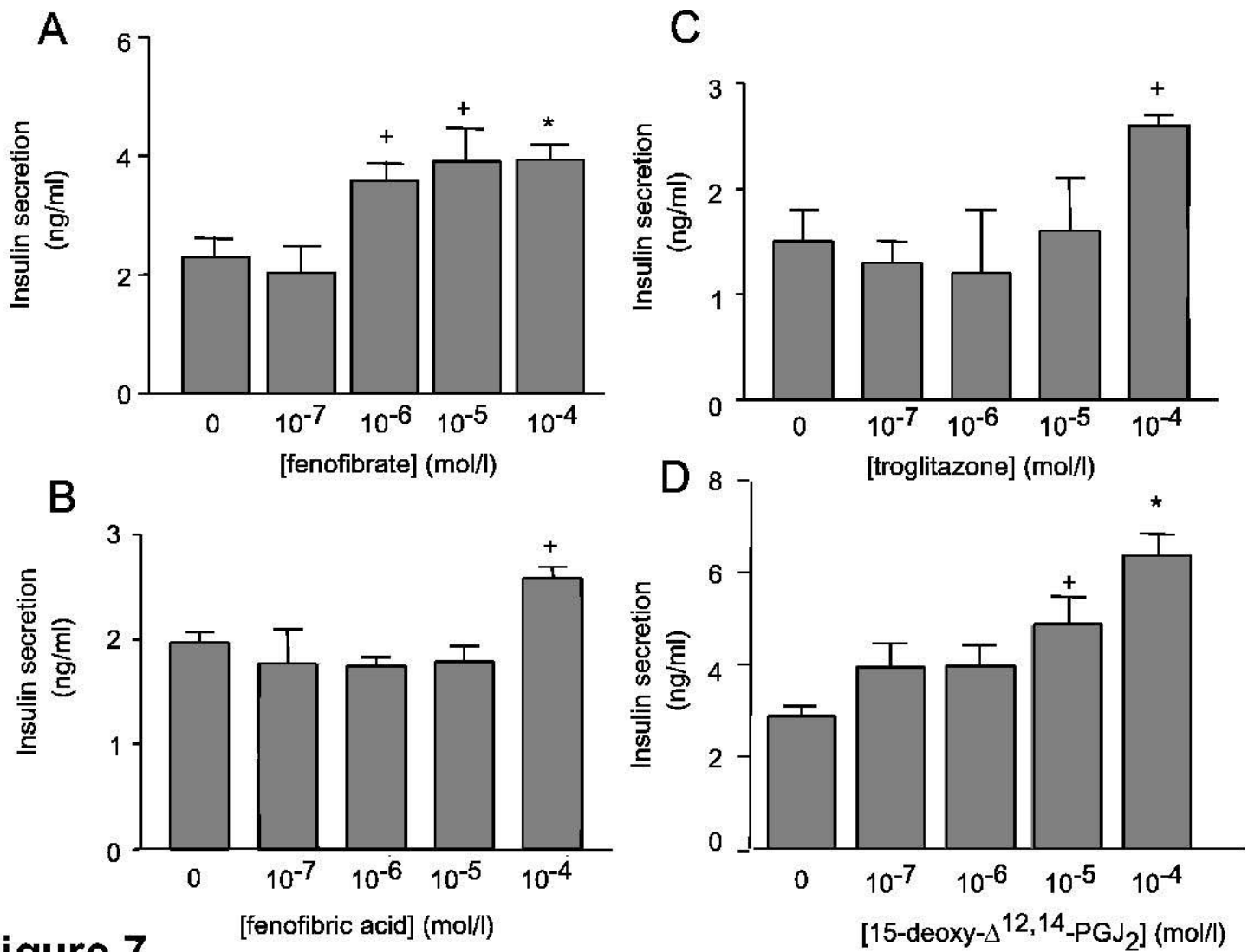
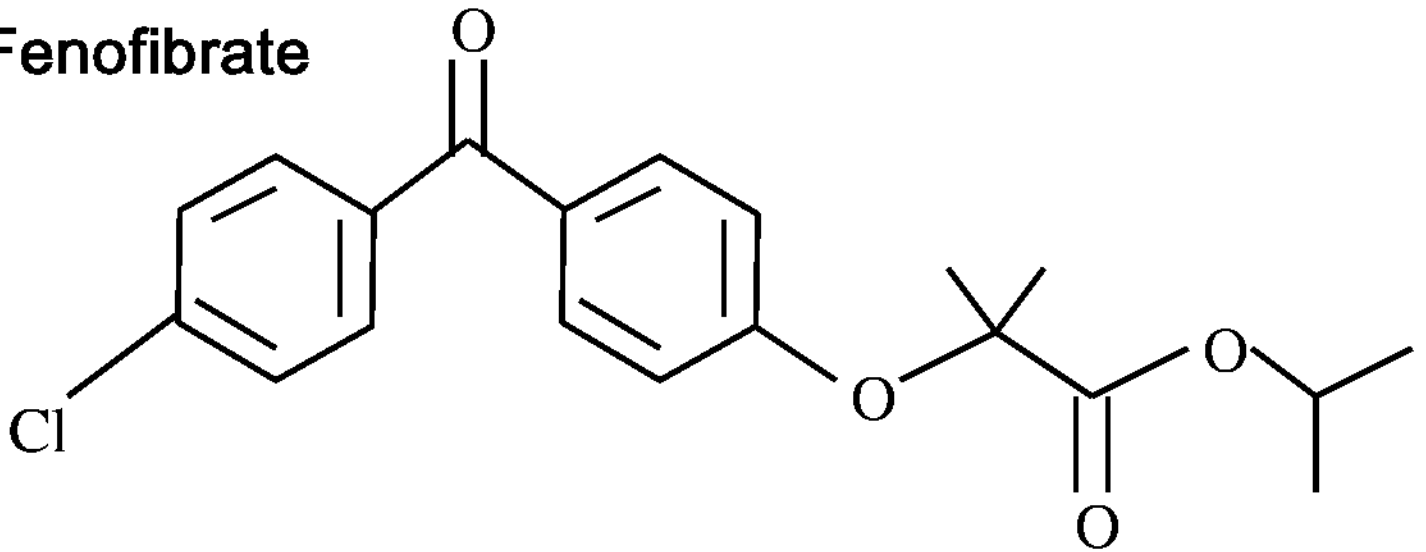


Figure 7

Fenofibrate



Fenofibric acid

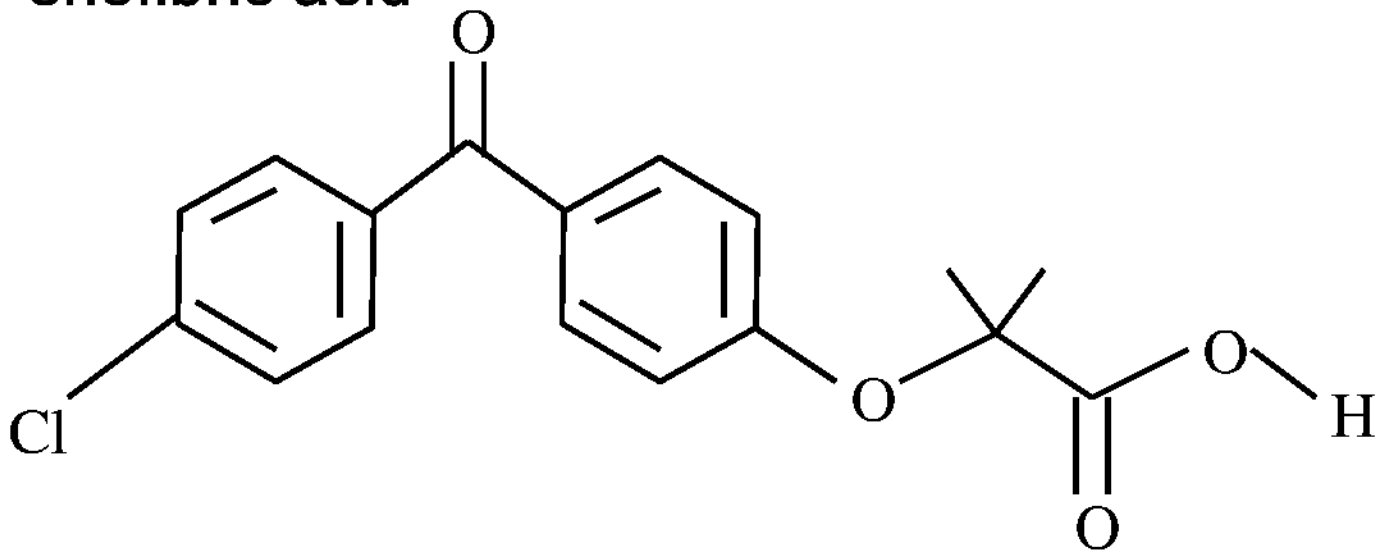


Figure 8