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Fenofibrate, troglitazone and 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 close K_{ATP}-channels and induce insulin secretion.

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Running title: K. Shimomura et al. PPAR independent effects of its ligands

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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 PPARα ligands on pancreatic β-cells also remain unclear. We investigated the effects of PPARα ligands (fenofibrate and fenofibric acid), a PPARγ ligand (troglitazone), and an endogenous ligand of PPARγ (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-$\Delta^{12,14}$-PGJ_2)) 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-$\Delta^{12,14}$-PGJ_2 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-$\Delta^{12,14}$-PGJ_2 showed half maximal inhibition of K_{ATP}-channel currents (IC_{50}) at 3.26 μmol /l, 94 μmol /l, 2.1 μmol /l and 7.3 μmol /l respectively. Fenofibrate ($\geq 10^{-6}$ mol /l), 15-deoxy-$\Delta^{12,14}$-PGJ_2 ($\geq 5 \times 10^{-5}$ mol /l) and troglitazone ($\geq 10^{-6}$ mol /l) inhibited [^{3}H]-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-$\Delta^{12,14}$-PGJ_2 ($\geq 10^{-5}$ mol /l) increased insulin secretion from HIT-T15 when applied for 10 min. Our data suggest that PPARα and γ ligands directly interact with the β-cell membrane and stimulate insulin secretion.

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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. PPARα is known to regulate fatty acid metabolism by controlling its oxidation, and PPARγ is known to involve in glucose homeostasis and adipocyte proliferation (Lemberger et al., 1996; Corton et al., 2000). Fenofibrate is a PPARα 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 PPARγ ligand, and 15-deoxy-

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$\Delta^{12,14}$-PGJ_2 is known to be an endogenous ligand of PPARγ (Kliewer et al., 1995)..

We have previously demonstrated that both troglitazone and pioglitazone, PPARγ ligands, stimulate acute-phase insulin secretion with a rapid increase of cytoplasmic Ca^{2+}, but inhibit chronic-phase insulin secretion in a Ca^{2+}-dependent manner (Ohtani et al., 1996; 1998). Removing extracellular Ca^{2+} abolished acute-phase insulin secretion by troglitazone and pioglitazone. The effects of PPARα 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 PPARα ligand (fenofibrate, fenofibric acid), a PPARγ ligand (troglitazone) and an endogenous ligand of PPARγ (15-deoxy-$\Delta^{12,14}$-PGJ_2) 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% O_2- 5% CO_2 incubator at 37^{o}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 $MgCl_2$, 2 mmol /l $CaCl_2$, 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 $MgCl_2$ and 1 mmol /l $CaCl_2$. 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 MΩ. 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-$\Delta^{12,14}$-PGJ_2 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 ^{o}C).

Binding experiments

HIT-T15 cells were resuspended at a density of 1.5 x 10 ^{7} cells /ml in glucose-free F-12K medium. Competitive inhibition assays were performed with 1.0 nmol /l [^{3}H]-glibenclamide, and various concentrations of fenofibrate, fenofibric acid, troglitazone, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 were incubated for 1hour 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 (Mutiscreeen 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 [^{3}H]-glibenclamide specific binding.

Insulin secretion study

For insulin secretion studies, cells were plated on 24-multiwell plates ($1x10^{5}$ 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-$\Delta^{12,14}$-PGJ_2.

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-$\Delta^{12,14}$-PGJ_2 (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-$\Delta^{12,14}$-PGJ_2 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:

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

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 μmol/l tolbutamide. [X] is the concentration of fenofibrate, fenofibric acid, troglitazone or 15-deoxy-$\Delta^{12,14}$-PGJ_2, 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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$$y = \frac{a-b}{1+(Ki/[X])^{h}} + b \qquad [2]$$

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-$\Delta^{12,14}$-PGJ_2, 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 ± 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 PPARα 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 x 10^{-4} mol /l of fenofibrate. The IC_{50} of inhibiting the K_{ATP} channel current by fenofibrate was 3.3 ± 0.5 μmol /l (n=8) and the Hill coefficient was 1.1 ± 0.2.

Effect of PPARα 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, irreversively 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 ± 0.5 μmol /l (n=8) and the Hill coefficient was 2.1±0.26.

Effect of PPARγ 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 irreversively 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 ± 0.4 μmol /l (n=8) and the Hill coefficient was 1.9 ± 0.32.

Effect of endogenous ligand of PPARγ (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 irreversively 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 ± 0.96 μmol /l (n=8) and the Hill coefficient was 1.0 ± 0.13.

Inhibition binding assay with PPARα ligands.

To examine the possibility that PPARα ligands may bind to the same site as glibenclamide, we performed an inhibition binding assay with 1.0 nmol /l [^{3}H]-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 x 10^{-7} mol /l (74.3 ± 3.2 % at 1 x 10^{-6} mol /l, 63.5 ± 1.8 % at 1 x 10^{-5} mol /l and 56.3 ± 4.3 % at 1 x 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 PPARγ ligands.

Similarly, PPARγ 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 x 10^{-6} mol /l (60.2 ± 0.1% at 5x10^{-6} mol /l, 50.0 ± 0.1% at 1x10^{-5} mol /l and 40.0 ± 0.01% at 5 x 10^{-5} mol /l, n=3) (Fig. 6A). Troglitazone inhibited [^{3}H]-glibenclamide binding at concentrations higher than 1 x 10^{-6} mol /l (79.9 ± 3% at 1 x 10^{-5} mol /l and 52.7 ± 6.5 % at 1 x 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 PPARα ligand (fenofibrate, fenofibric acid) and PPARγ 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} ~ 10^{-4} mol /l) (Fig 7C) and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (10^{-7} ~ 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 PPARγ ligand, troglitazone, for 10 min also enhanced insulin secretion. Under drug-free conditions insulin secretion was 1.5 $\pm$ 0.3 ng/ml (n=8), whilst in the presence of 10^{-4} mol/l Troglitazone, insulin secretion was 2.6 $\pm$ 0.1 ng/ml (n=8, Fig 7C).

The effects of the endogenous PPARγ ligand 15-deoxy-$\Delta^{12,14}$-PGJ_2 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-$\Delta^{12,14}$-PGJ_2 increased secretion to 4.9 ± 0.6 ng /ml and 10^{-4} mol /l 15-Deoxy-$\Delta^{12,14}$-PGJ_2 to 6.4 ± 0.5 ng /ml (Fig. 7D).

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Discussion

The present studies demonstrated that both PPARα ligands (fenofibrate, fenofibric acid) and PPARγ ligands (troglitazone, 15-deoxy-$\Delta^{12,14}$-PGJ_2) inhibit K_{ATP} channel and induce acute-phase insulin secretion. Fenofibrate, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and troglitazone inhibited the binding of [^{3}H]-glibenclamide. These results indicated that PPARα 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-$\Delta^{12,14}$-PGJ_2, 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-$\Delta^{12,14}$-PGJ_2 may inhibit the K_{ATP} channel in a similar manner to glibenclamide. In addition, the fact that fenofibrate, troglitazone and 15-deoxy-$\Delta^{12,14}$-PGJ_2 inhibit the binding of [^{3}H]-glibenclamide suggests that these PPAR ligands may interact directly with SUR. Therefore, we speculate that fenofibrate, troglitazone, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 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 [^{3}H]-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 [^{3}H]-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 [^{3}H]-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-$\Delta^{12,14}$-PGJ_2 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 administrating 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 IC_{50} 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 PPARα agonist, WY 14,643 opposed insulin secretion elicited by high fat feedings in rats. Tordjman et al (2002) also reported that over expression of PPARα in INS-1 cells, an insulinoma cell line, decreased basal and glucose-stimulated insulin secretion. These two reports clearly demonstrated that chronically activating PPARα 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 PPARα ligands may be produced by the mechanism of PPARα 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 PPARγ ligands such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 and troglitazone. These include effects caused by

on microvascular endothelial cells and skeletal muscle (Brunmair et al., 2001; Jozkowicz et al., 2001). PPARγ-independent effects of 15-deoxy-$\Delta^{12,14}$-PGJ_2 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-$\Delta^{12,14}$-PGJ_2 has a direct effect on the IκB kinase complex and NF-κB activation in HeLa cells, which do not express PPARγ (Rossi et al., 2000). It was also reported that 15-deoxy-$\Delta^{12,14}$-PGJ_2 impairs cytokine signaling by inhibiting IκB, stimulating IFN-γ, and nitric oxide generation independently of PPARγ 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 μmol/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^+ (Kv) channels and ATP-dependent Ca^{2+} 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 PPARγ, 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 PPARγ (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 μ mol /l, h=1.14. Values represent means ± 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 μ mol /l, h=2.1. Values represent means ± 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 μ mol /l, h=1.9. Values represent means ± standard errors. Asterisk * indicate $p<0.01$ vs. control. n=8 in each group.

Fig. 4. Effect of 15-deoxy-Δ^{1214}-PGJ_2 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 $4x10^{-5}$ mol/l 15-deoxy-Δ^{1214}-PGJ_2 **(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}-PGJ_2. Relationship between the 15-deoxy-Δ^{1214}-PGJ_2 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 μmol/l, h=1.0. Values represent means ± standard errors. Asterisk * indicate $p<0.01$ vs. control. n=8 in each group.

Fig. 5. Inhibition of the specific [^{3}H]-glibenclamide binding to HIT-T15 cells by increasing concentrations of unlabelled fenofibrate (**A**) and fenofibric acid (**B**). Fenofibrate inhibited the binding of [^{3}H]-glibenclamide at higher concentrations than the unlabelled glibenclamide whilst fenofibric acid had no effect. Values represent the means ± 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 [^{3}H]-glibenclamide binding to HIT-T15 cells by increasing concentrations of unlabelled 15-deoxy-Δ^{1214}-PGJ_2 (**A**) and troglitazone (**B**). 15-deoxy-Δ^{1214}-PGJ_2 and troglitazone inhibited the binding of [^{3}H]-glibenclamide at higher concentrations than the unlabelled-glibenclamide. Values represent the means ± 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}-PGJ_2 **(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}-PGJ_2. The glucose concentration in the medium used in this study was 7mmol /l. Values represent means ± 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.

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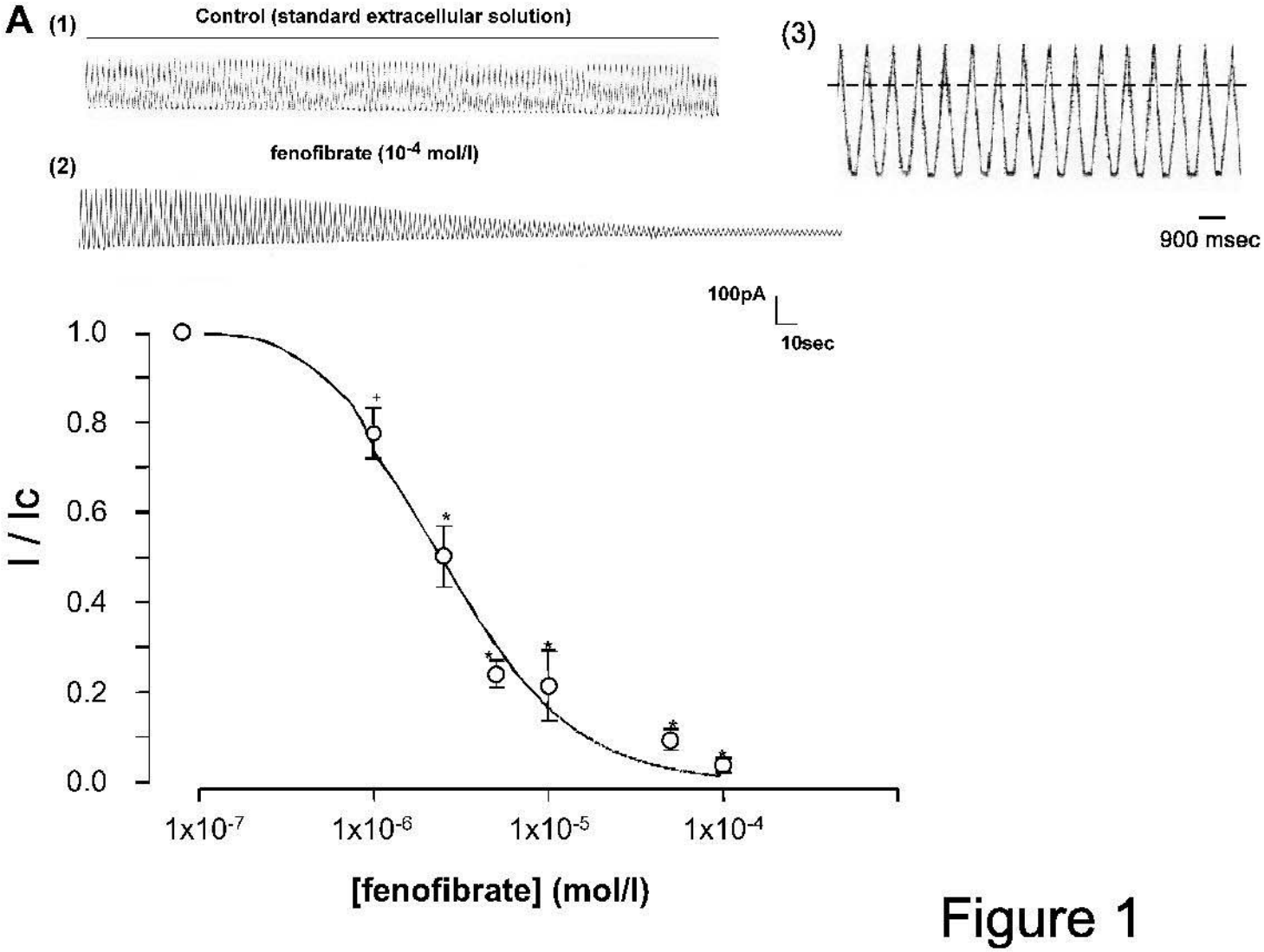

Figure 1

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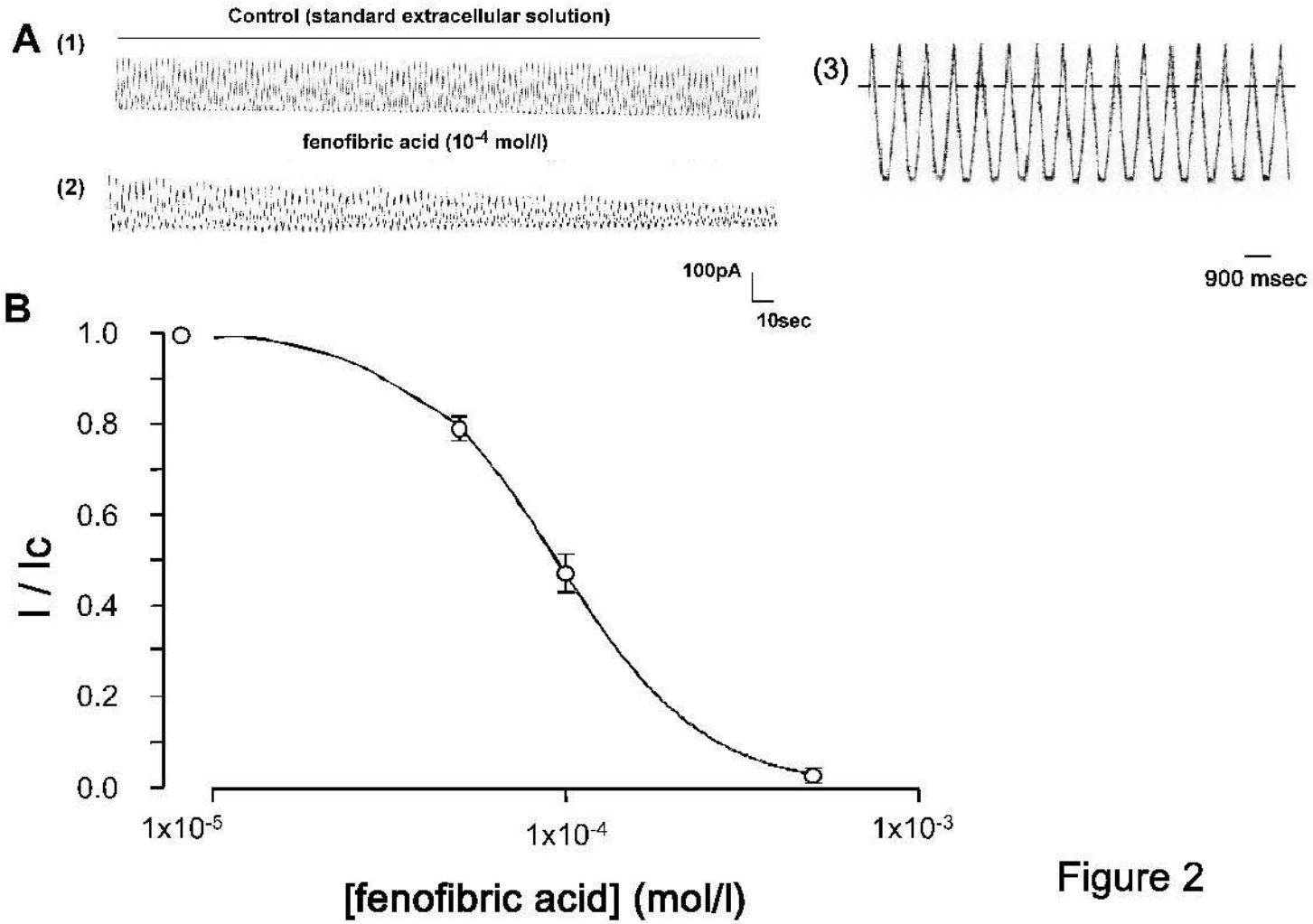

Figure 2

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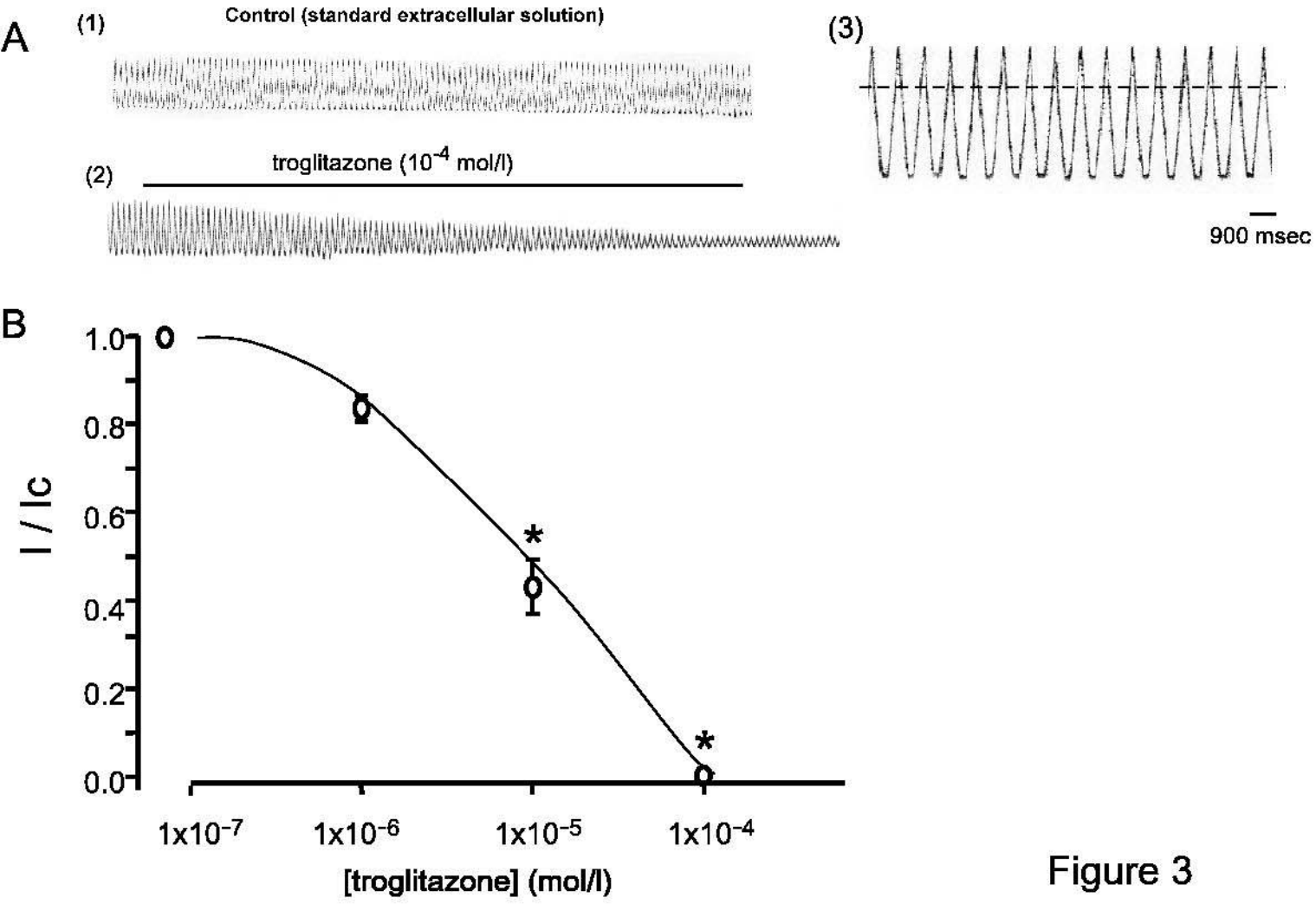

Figure 3

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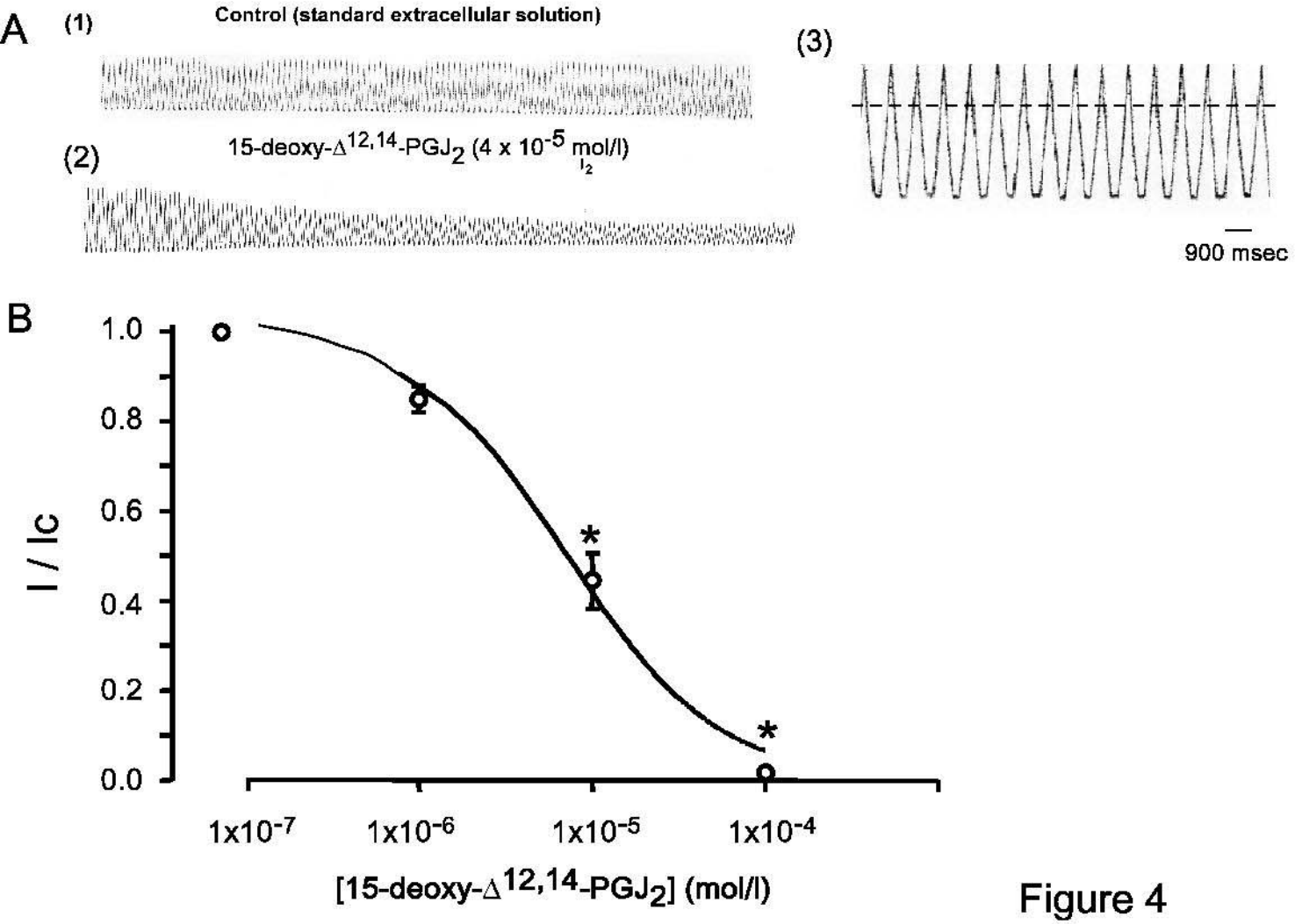

Figure 4

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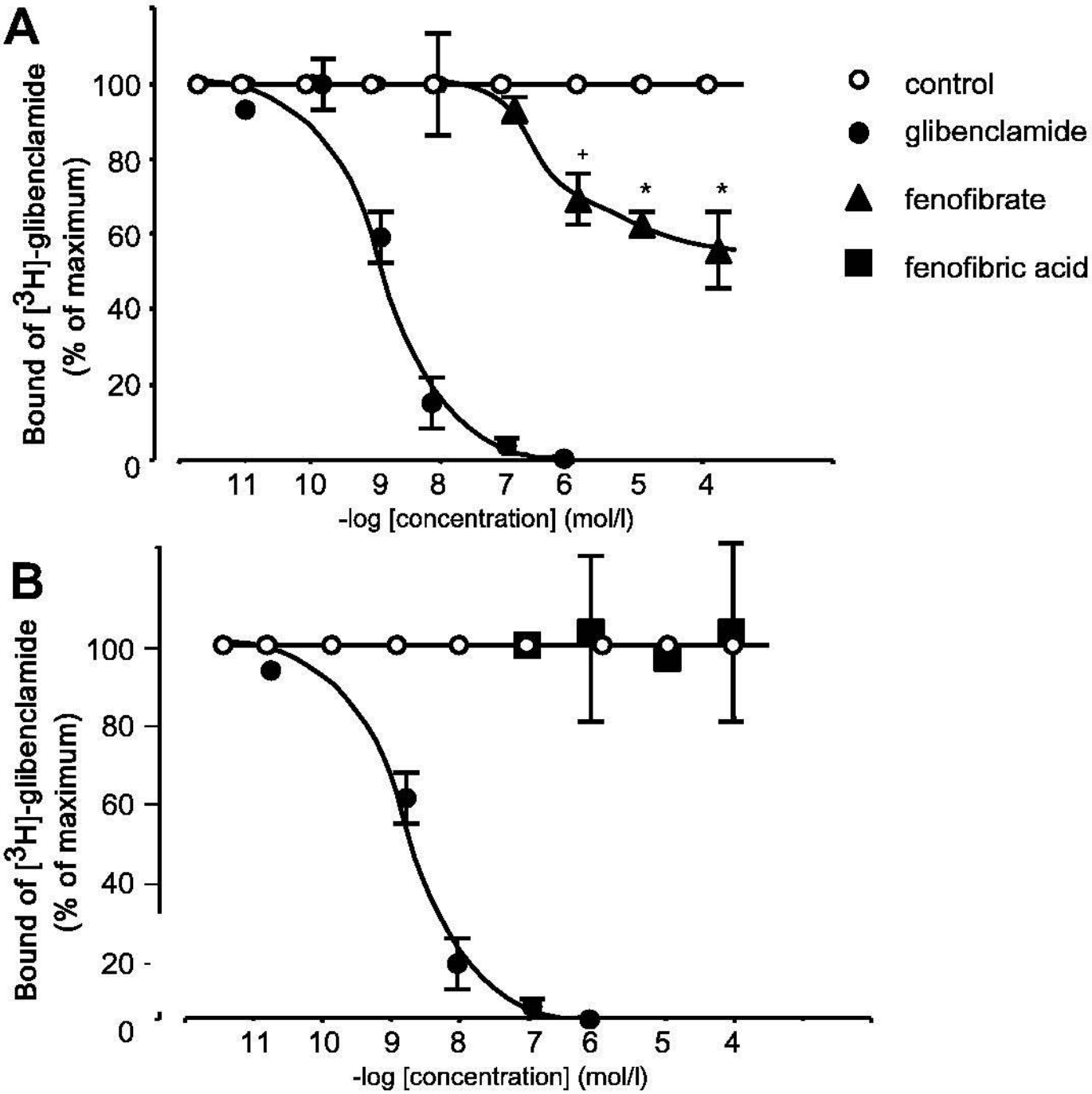

Figure 5

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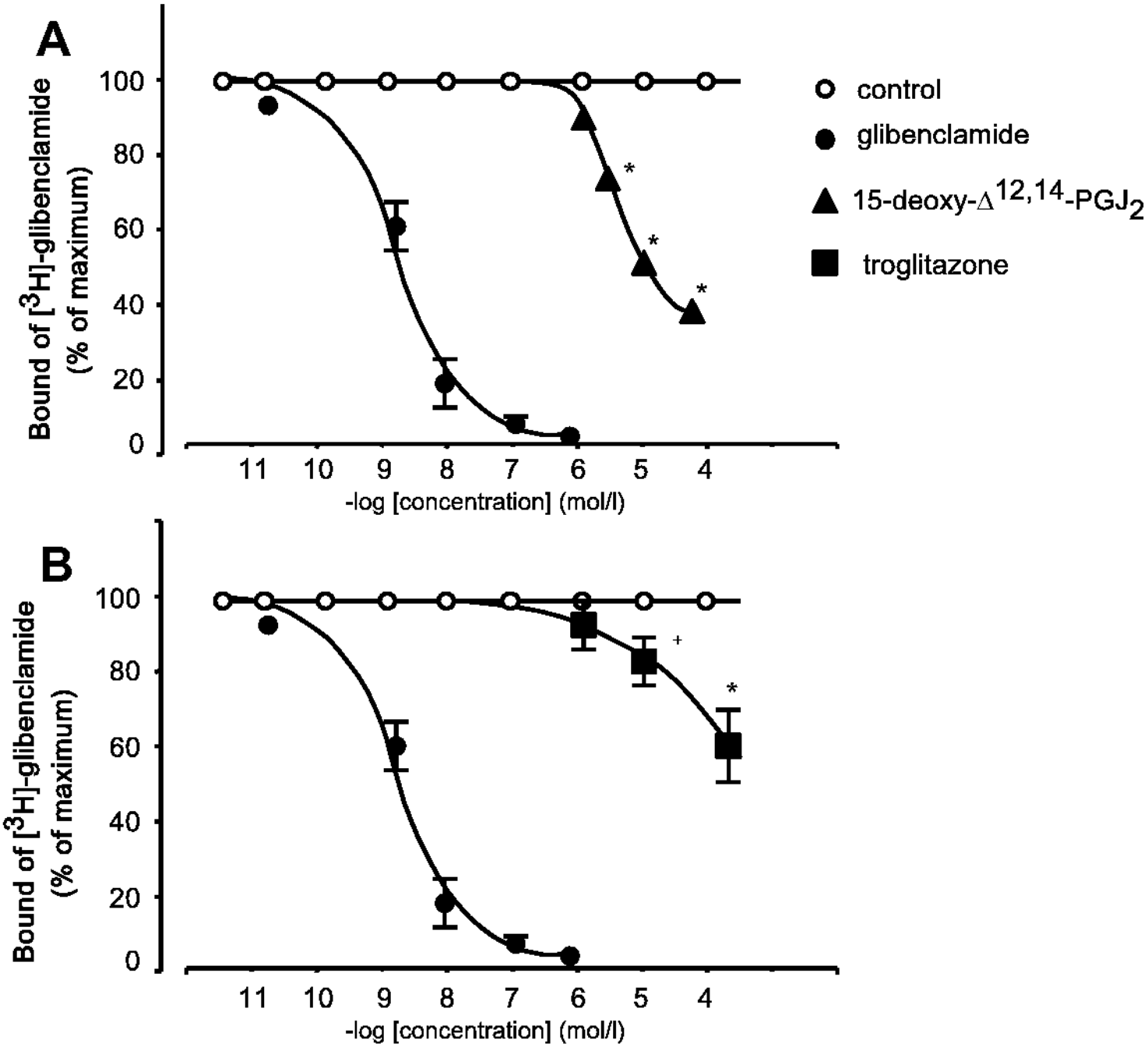

Figure 6

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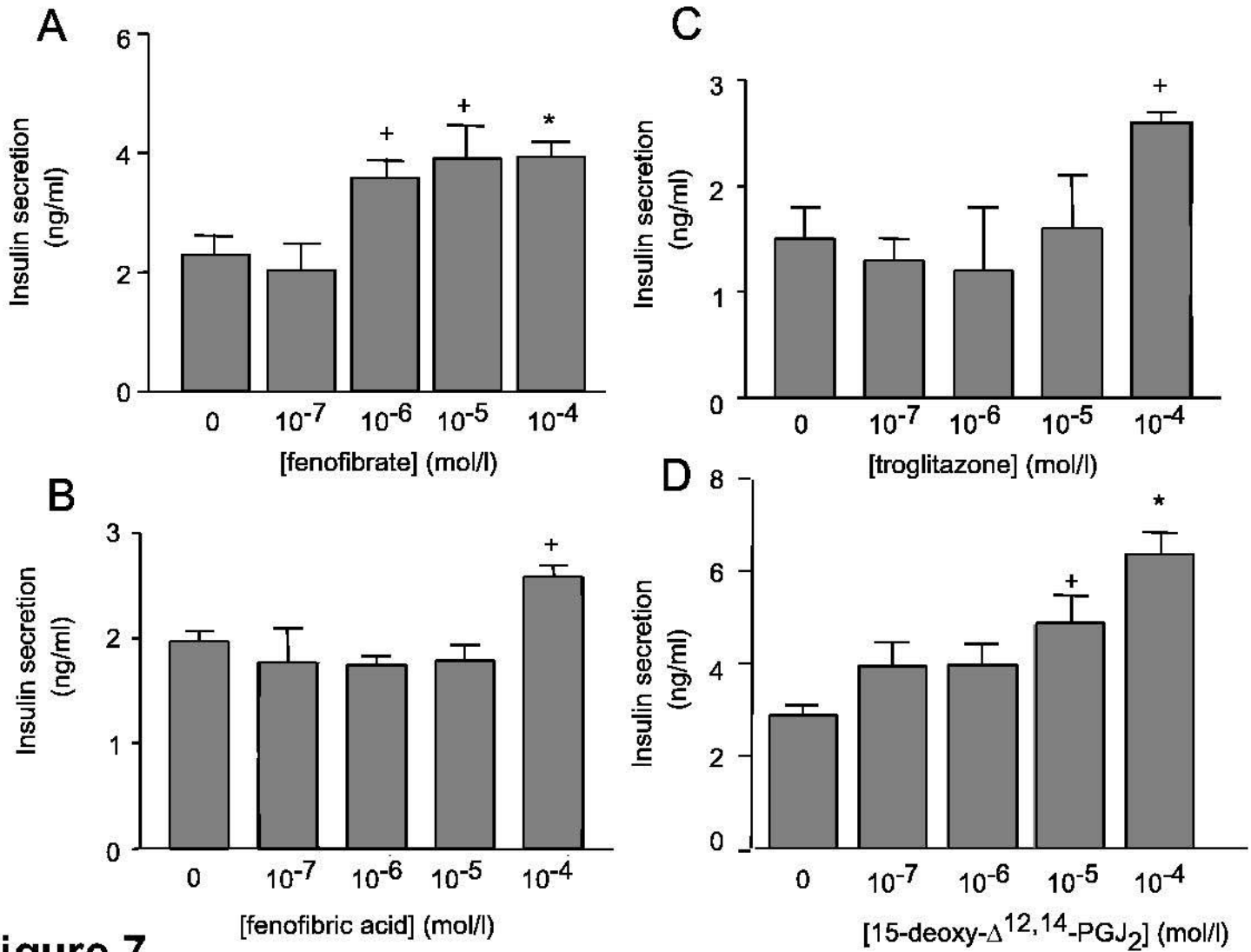

Figure 7

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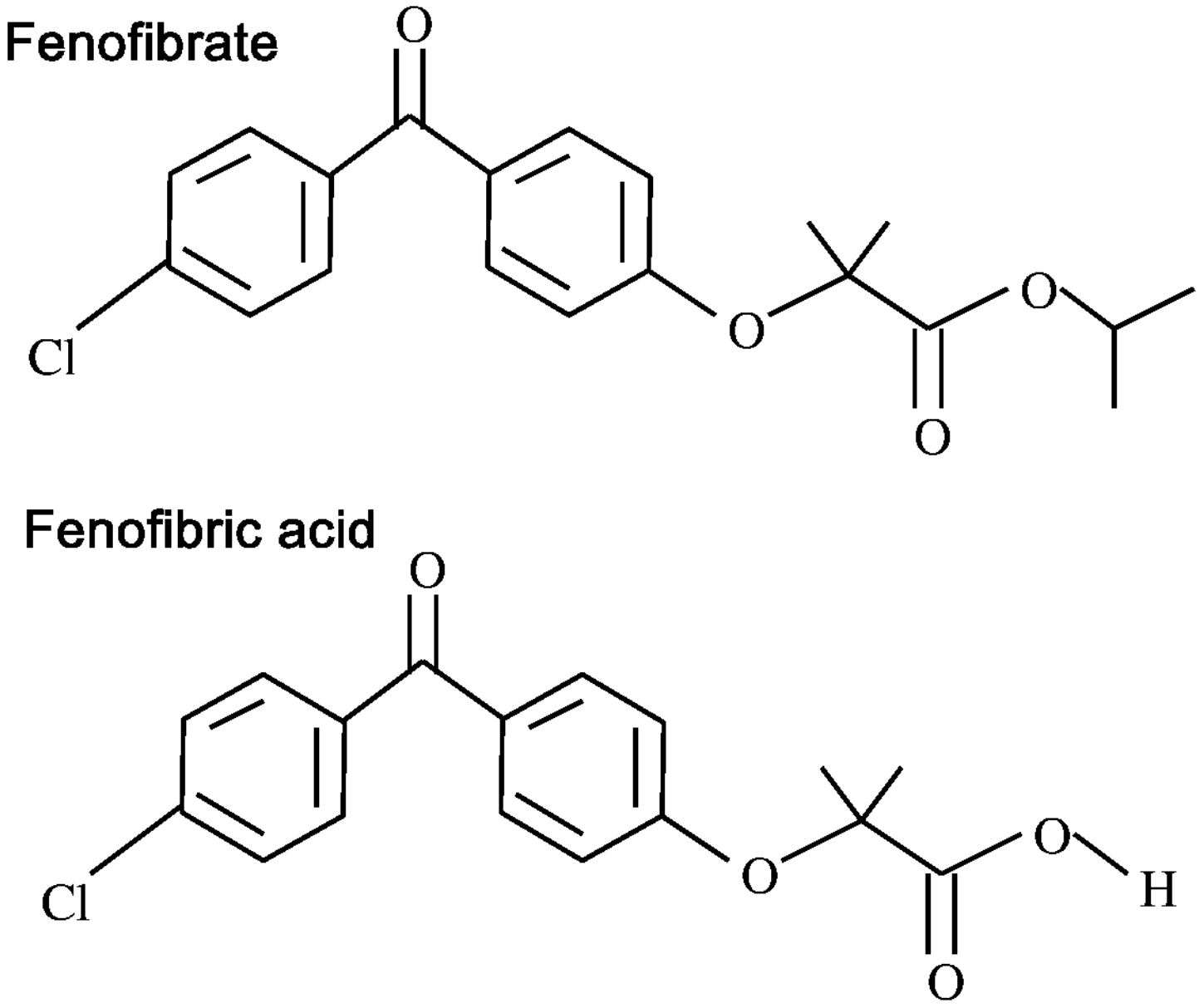

Figure 8