TheMechanismsofInsulinSecretionandCalciumSignalinginPancreaticβ-cellsExposedtoFluoroquinolones

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Fluoroquinolones reportedly induce hypoglycemia through stimulation of insulin secretion from pancreatic β-cells via inhibition of KATP channels and activation of L-type voltage-dependent Ca2+ channels. In physiological condition, the cytosolic Ca2+ concentration ([Ca2+]c) is also regulated by release of Ca2+ from intracellular Ca2+ stores. In this study, we investigated the mechanism of insulin secretion induced by fluoroquinolones, with respect to intracellular Ca2+ stores. Even where the absence of supplemental extracellular Ca2+, insulin secretion and [Ca2+]c were increased by gatifloxacin, levofloxacin or tolbutamide. Insulin secretion and the rise of [Ca2+]c induced by fluoroquinolones were reduced by depleting of Ca2+ in endoplasmic reticulum (ER) by thapsigargin, and inhibiting ryanodine receptor of ER by dantrolene. Inhibition of insulinostol 1,4,5-triphosphate receptor of ER by xestospongin C suppressed insulin secretion induced by fluoroquinolones, whereas it did not affect [Ca2+]c. Destruction of acidic Ca2+ stores such as lysosome and lysosome-related organelles by glycy1-phenylalanine-2-naphthylamide (GPN) did not affect insulin secretion and the rise of [Ca2+]c induced by fluoroquinolones. The increase in insulin and [Ca2+]c induced by tolbutamide were reduced by thapsigargin, dantrolene, and GPN but not by xestospongin C. In conclusion, fluoroquinolones induces Ca2+ release from ER mediated by the ryanodine receptor, and the reaction might involve in insulin secretion. Sulfonylureas induce Ca2+ release from GPN-sensitive acidic Ca2+ stores, but fluoroquinolones did not.

Key words fluoroquinolone; insulin; calcium signaling; hypoglycemia; pancreatic β-cell

Fluoroquinolones are frequently used for the treatment of various infectious diseases because of their broad antimicrobial spectrum and their positive safety profile. However, some fluoroquinolones, such as temafloxacin and gatifloxacin, were withdrawn from the U.S. market because they cause a severe drop in blood glucose levels. Other fluoroquinolones, such as levofloxacin, ciprofloxacin, and moxifloxacin have also been reported to cause hypoglycemia. It has been suggested that the observed hypoglycemia was associated with elevated levels of fluoroquinolones in the blood, and that the advanced age or renal dysfunction, which can also cause high levels of fluoroquinolones in the blood, are risk factors for development of hypoglycemia.

It has been indicated that some fluoroquinolones induce insulin secretion from pancreatic β-cells in a concentration-dependent manner. Insulin secretion from β-cells induced by glucose or sulfonylureas is closely related to the rise of cytosolic Ca2+ concentration ([Ca2+]c). An elevation in [Ca2+]c was also observed when fluoroquinolones stimulated insulin secretion in β-cells, and it was suggested to be associated with Ca2+ influx via inhibition of KATP channels and activation of L-type voltage-dependent Ca2+ channels (VDCC). On the other hand, intracellular Ca2+ stores have also been contributed to physiological insulin secretion. The purpose of this study was to clarify the intracellular calcium dynamics in β-cells when insulin secretion was induced by fluoroquinolones.

MATERIALS AND METHODS

Reagents Gatifloxacin and levofloxacin were purchased from LKT Laboratories (St. Paul, MN, U.S.A.), Tolbutamide and glycy1-phenylalanine-2-naphthylamide (GPN) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Thapsigargin was purchased from Alomone Labs (Jerusalem, Israel), and dantrolene and xestospongin C were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chemicals were dissolved in dimethylsulfoxide or 100 mM sodium hydroxide to obtain stock solutions, and diluted to the final concentration before use. When 100 mM sodium hydroxide was used as a solvent, the solution was neutralized with 100 mM hydrochloric acid before use. Other materials without description were commercially available reagents of analytical grade.

Cell Culture The insulin secreting cell line HIT-T15, derived from Syrian hamster pancreatic islet cells, was purchased from DS Pharma Biomedical (Osaka, Japan). The cells (passage numbers 74–83) were cultured under humidified conditions (with 5% CO2 at 37°C) in Ham's F12K medium (Sigma-Aldrich) supplemented with 2.5 µg/mL NaHCO3, 10% fetal bovine serum (Nichirei Bioscience, Tokyo, Japan), 100IU/mL penicillin G, and 0.1 mg/mL streptomycin (Sigma-Aldrich).

Insulin Secretion HIT-T15 cells (1.0×10^6 cells) were seeded in 24-well plates and cultured for 72 h before the assay. The cells were preincubated in Hank’s balanced salts (HBSS) (Sigma-Aldrich)-N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (137 mM NaCl, 5.4 mM...
KCl, 0.8 mM MgSO₄, 0.43 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 8 mM HEPES, 10 mM glucose, 0.1% bovine serum albumin (BSA), pH 7.4) with or without 1.3 mM CaCl₂, containing 1 μM thapsigargin, 10 μM dantrolene, 2 μM xestospongion C, or 50 μM GPN at 37°C for 30 min. The solution was then changed to the buffer containing 100 μM gatifloxacin, 100 μM levofloxacin, or 400 μM tolbutamide and incubated for another 60 min under the same conditions. After incubation, the insulin concentration of the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available assay kit (Morinaga Institute of Biological Science, Kanagawa, Japan). Residual cells were lysed with 300 mM sodium hydroxide. After neutralization with 300 mM hydrochloric acid, the protein concentration was determined by Lowry’s method. Cytosolic Ca²⁺ Concentration HIT-T15 cells (5.0 × 10⁵ cells) were seeded in 35-mm dishes, and cultured for 24 h before the assay. The cells were incubated in HBSS-HEPES buffer with or without 2.5 mM CaCl₂, supplemented with 5 μM Fluo-3/AM (Dojindo Laboratories, Kumamoto, Japan), a cytosolic calcium indicator, for 30 min in the absence of light. The solution was then changed to a buffer without Fluo-3/AM, and the cells were preincubated with the buffer containing 1 μM thapsigargin, 10 μM dantrolene, 2 μM xestospongion C, or 50 μM GPN for 30 min under the same conditions. After preincubation, the buffer was exchanged and [Ca²⁺]ₗ was measured for 40 min. After 3 min, 100 μM gatifloxacin, 100 μM levofloxacin, or 400 μM tolbutamide was added to the buffer. [Ca²⁺]ₗ was determined by the fluorescence intensity of Fluo-3 (Ex. 488 nm, Em. 530 nm) measured using a high-speed intracellular Ca²⁺ measurement system, ARGUS-HiSCA (Hamamatsu Photonics, Shizuoka, Japan). Each value represents the ratio of the measured intensity to the initial measured intensity.

Role of Extracellular Ca²⁺ Influx and Intracellular Ca²⁺ Stores on Insulin Secretion To examine the effects of extracellular Ca²⁺ concentration on insulin secretion and the change of [Ca²⁺]ₗ induced by fluoroquinolones, insulin secretion and [Ca²⁺]ₗ were assessed with or without extracellular Ca²⁺.

Next, to examine the effects of the release of Ca²⁺ from intracellular stores, 1 μM of thapsigargin, a sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor, 10 μM of dantrolene, a ryanodine receptor (RyR) inhibitor, or 2 μM of xestospongion C, an inositol 1,4,5-trisphosphate receptor (IP₃R) inhibitor, was used. Finally, the involvement of Ca²⁺ release from acidic Ca²⁺ stores was examined with 50 μM of GPN, which lyses acidic Ca²⁺ stores such as lysosomes and lysosome-related organelles (LROs) which both express cathepsin C. All these compounds were tested in the presence of extracellular Ca²⁺, with the exception of thapsigargin.

Statistics Each value is presented as the mean±standard deviation (S.D.). The Student’s t-test, Dunnett’s test, and Bonferroni–Dunn’s test were used to test the differences among the means. A value of p<0.05 was considered statistically significant.

RESULTS

Gatifloxacin, levofloxacin, or tolbutamide induced insulin secretion from the HIT-T15 cells in a concentration-dependent manner (data not shown). Fluoroquinolones induced a rise in [Ca²⁺]ₗ similar to that by tolbutamide. The changes in [Ca²⁺]ₗ were greater with the presence of extracellular Ca²⁺ (Figs. 1A, B). Similarly, extracellular Ca²⁺ augmented insulin secretion induced by fluoroquinolones or tolbutamide (Fig. 1C). The [Ca²⁺]ₗ slightly increased by either fluoroquinolones or tolbutamide under the absence of supplemental Ca²⁺, but this increase was suppressed by thapsigargin (Figs. 2A, B). Insulin secretion induced by fluoroquinolones or tolbutamide also slightly reduced by thapsigargin (Fig. 2C).

In presence of extracellular Ca²⁺, dantrolene reduced the rise of [Ca²⁺]ₗ, and insulin secretion induced by fluoroquinolones or tolbutamide (Figs. 3A, B, G). The levels of [Ca²⁺]ₗ did not change in response to xestospongion C, but insulin secretion induced by fluoroquinolones was reduced by xestospongion C in the presence of extracellular Ca²⁺. However, the insulin secretion induced by tolbutamide was not affected by xestospongion C (Figs. 3C, D, G). Also, GPN significantly reduced the rise of [Ca²⁺]ₗ, or insulin secretion induced by tolbutamide, but the rise of [Ca²⁺]ₗ, and insulin secretion induced by fluoroquinolones were not affected (Figs. 3E–G).

DISCUSSION

Hypoglycemia caused by fluoroquinolones is a serious clinical issue. Fluoroquinolones stimulate insulin secretion from pancreatic β-cells in glucose-dependent and concentration-dependent manner, and our experimental system using HIT-T15 cell lines also demonstrated similar results. In our report, levofloxacin significantly induced insulin secretion, but the former study reported that levofloxacin would not induce significant increase of insulin secretion from mouse pancreatic islets. Although the intensity was varied, insulin secretion was generally observed in fluoroquinolones. And actually, levofloxacin was demonstrated to induce insulin secretion in rat model, and hypoglycemia caused by levofloxacin was noteworthy clinical problems. This contradiction might be due to the difference of experimental conditions.

The insulin secretion induced by gatifloxacin or levofloxacin was significantly higher in the presence of extracellular Ca²⁺, which suggests that the influx of Ca²⁺ was important for insulin secretion induced by fluoroquinolones, similar to that by sulfonfonylureas as already reported. Fluoroquinolone-induced insulin secretion was described as by inhibiting the activity of K_{ATP} channels, similar to that by sulfonfonylureas. However, [Ca²⁺]ₗ was increased by gatifloxacin and levofloxacin in the absence of supplemental Ca²⁺, so it was thought that other sources of Ca²⁺ should also play roles in insulin secretion induced by fluoroquinolones. To test this hypothesis, we examined the effects of inhibition of Ca²⁺ release from intracellular stores.

The rise of [Ca²⁺]ₗ by gatifloxacin or levofloxacin was suppressed by depleting Ca²⁺ in endoplasmic reticulum (ER) by SERCA inhibitor thapsigargin, which suggests that Ca²⁺ release from the ER should be involved. Insulin secretion by gatifloxacin or levofloxacin was also reduced by thapsigargin, though the differences were not significant. These experiments just conducted without supplemental Ca²⁺ in reaction buffer without chelating intracellular Ca²⁺. Treatment with thapsigargin almost entirely suppressed the rise of [Ca²⁺]ₗ, so the effects of residual Ca²⁺ might be restrictive. However,
influences of Ca\textsuperscript{2+} stored in other than ER, such as micro-domains nearby plasma membrane, might not be ruled out.

In addition, the rise of [Ca\textsuperscript{2+}]\textsubscript{c} induced by gatifloxacin or levofloxacin was significantly reduced by ryr inhibitor dantrolene but not by IP\textsubscript{3} inhibitor xestospongin C. Thus, the Ca\textsuperscript{2+} release from ER induced by fluoroquinolones likely occurred by the activation of the RyR rather than the IP\textsubscript{3}. However, because of nonspecific actions of inhibitors, more experiments using knockdown models of RyR should be necessary.
Fig. 3. Effect of Dantrolene, Xestospongin C, or Glycyl-l-phenylalanine-2-naphthylamide (GPN) on [Ca$^{2+}$]$_c$ and Insulin Secretion in HIT-T15 Cells Exposed to Gatifloxacin, Levofloxacin, or Tolbutamide

After loading Fluo-3/AM, cells were incubated for 40 min in HBSS-HEPES buffer with or without 10 µM dantrolene, 2 µM xestospongin C, or 50 µM GPN. The buffer was supplemented with 100 µM gatifloxacin, 100 µM levofloxacin, or 400 µM tolbutamide 3 min after the start of incubation (A–F). Cells were incubated for 60 min in HBSS-HEPES buffer with or without 10 µM dantrolene, 2 µM xestospongin C, or 50 µM GPN containing 100 µM gatifloxacin, 100 µM levofloxacin, or 400 µM tolbutamide (G). Each value is presented as the mean±S.D. (A–F; n=10, G; n=4) *p<0.05, **p<0.01 vs. gatifloxacin, levofloxacin, or tolbutamide (A–F) †p<0.05, ‡p<0.01 vs. without inhibitors (G).
to clarify this hypothesis. Curiously, insulin secretion induced by gatifloxacin or levofloxacin was significantly reduced by xestospongin C in contrast to [Ca^{2+}]_c. This discrepancy was not observed with tolbutamide. Though more investigations are needed, this result indicated that IP_3 have some particular roles on insulin secretion induced by fluoroquinolones.

Experiments involving GPN, which destructs acidic Ca^{2+} stores such as lysosomes and LROs, indicated that gatifloxacin and levofloxacin might not affect GPN-sensitive acidic Ca^{2+} stores. Sulfonylureas were reported to activate the cAMP-regulated guanine nucleotide exchanger factor 2 which stimulates the synthesis of nicotinic acid adenine dinucleotide phosphate (NAADP), the potential messenger for Ca^{2+} release from acidic Ca^{2+} stores. In this experiment, insulin secretion and the rise of [Ca^{2+}]_c induced by tolbutamide were significantly reduced by GPN, despite the depletion of Ca^{2+} in ER by thapsigargin completely suppressed the rise of [Ca^{2+}]_c induced by tolbutamide under the condition without supplemental Ca^{2+}. These results suggested that tolbutamide-induced Ca^{2+} release from the acidic Ca^{2+} stores might be associated with ER. Unlike sulfonylureas, fluoroquinolones would not cause increase in [Ca^{2+}]_c via release from GPN-sensitive acidic Ca^{2+} stores.

In this report, [Ca^{2+}]_c values were decreased from baselines, when the buffer containing test reagents were added. But the similar results were also observed with control, it might be due to interference of the fluorescence caused by increase of buffer volume or ripple of buffer surface. The immediate increase of [Ca^{2+}]_c, observed after adding tolbutamide with supplemental Ca^{2+}, was not occurred with gatifloxacin or levofloxacin. Former studies demonstrated similar immediate reaction by fluoroquinolones. These differences might have some significance, but the certain cause of the discrepancy was obscure. Additionally, in these [Ca^{2+}]_c experiments, 2.5 mm of supplemental extracellular Ca^{2+}, higher than the experiment of insulin secretion, was used in order to observe clear reactions. Although the possibility that insulin secretion and [Ca^{2+}]_c, were not reproduced at same condition could be undeniable. But we considered these data were evaluable by comparison with that of tolbutamide.

In conclusion, we found that insulin secretion induced by fluoroquinolones is resulted from Ca^{2+} release from intracellular Ca^{2+} stores, especially ER mediated by RyR, as well as extracellular Ca^{2+} entry in pancreatic β-cells. However, fluoroquinolones have no effects on the GPN-sensitive acidic Ca^{2+} stores, which are involved in increasing [Ca^{2+}]_c in response to sulfonylureas.

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REFERENCES