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The Synergistic Effects of Pioglitazone on the Glucose-Lowering Action of Metformin in Relation to OCT1 and Gluts m-RNA Expression in Healthy Volunteer

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Abstract

Objective: Organic Cation Transporter 1 (OCT1) plays a key role in the disposition of metformin in hepatocytes; a recent non-clinical study reported that the peroxisome-proliferator activated receptor γ agonist pioglitazone increased the expression of Slc22a1 (Oct1) by 3.1-fold as well as its transporting function. We therefore evaluated the effect of pioglitazone on the glucose-lowering effect of metformin in 15 human participants using the Oral Glucose Tolerance Test (OGTT) and measuring the mRNA levels of OCT1, as well as those of Glucose Transporter 1 (GLUT 1) and GLUT4, which are also important in glucose utilization, in peripheral blood cells.

Research design and methods: The glucose-lowering effects of metformin were evaluated by the OGTT before and after metformin treatment on Days 1 and 2 of the study and again on Days 18 and 19 after a 14-day course of pioglitazone 30 mg/day. Differences in maximum glucose levels (ΔGmax) and the areas under the glucose-time curve during the first 60 min after glucose ingestion (ΔAUCgluc60) and the entire 180-min test (ΔAUCgluc) caused by metformin treatment were determined before and after pioglitazone administration.

Results: Pioglitazone increased ΔGmax by 50.0% (P=0.021), ΔAUCgluc60 by 88.1% (P=0.020), and ΔAUCgluc by 266%. Pioglitazone did not increase OCT1 and GLUT1 mRNA levels in peripheral blood cells.

Conclusion: OCT1 induction plays a limited role in the synergistic effect of pioglitazone on the glucose-lowering activity of metformin. However, this synergistic effect lasted 3 days after pioglitazone treatment ended, which warrants further clinical investigation.

Keywords: Metformin; Pioglitazone; Peripheral blood cells

Introduction

Diabetes mellitus is a chronic and progressive disease, and type 2 diabetes, characterized by insulin deficiency and resistance, accounts for approximately 85 to 95% of all cases [1]. It is important to control glucose levels in diabetic patients to prevent complications and improve their quality of life [2]. Metformin is the first-line oral hypoglycemic drug for the treatment of type 2 diabetes; it lowers plasma glucose levels by inhibiting gluconeogenesis (3). Its pharmacokinetic distribution and elimination processes are mediated by Organic Cation Transporters (OCTs) [4,5]. OCT1 is primarily located in hepatocyte sinusoidal membranes [6], whereas OCT2 is mainly localized in the basolateral membrane of the kidney proximal tubule [7]. Metformin uptake by OCTs in the liver and kidney plays an important role in its pharmacokinetics and pharmacodynamics [8,9].

Pioglitazone is an antihyperglycemic agent that, in the presence of insulin resistance, increases hepatic and peripheral insulin sensitivity through the activation of peroxisome-proliferator activated receptor (PPAR)-γ in liver, fat, and skeletal muscle cells, increases peripheral and splanchnic glucose uptake, and decreases hepatic glucose output [10]. Combination therapy with metformin and pioglitazone is one of the most common regimens to control glucose levels in diabetic patients [11]. This combination produced better outcomes than metformin monotherapy in many studies [12]. However, it is unclear whether the better outcome is due to a synergistic or additive effect between the two oral hypoglycemic agents. In a non-clinical study, pioglitazone increased the expression of Slc22a1 (Oct1) by 3.1-fold in mice and increased [14C] tetraethylammonium bromide uptake in

H35 cells by 46% [13]. This indicates that pioglitazone may increase the uptake of metformin into the liver, the main action site of metformin in humans. Pioglitazone has also been reported to increase the glucose-lowering effect of agents by enhancing glucose transporter 1 (GLUT 1)- and GLUT4-mediated glucose uptake into cells [14,15].

In the present study, we hypothesized that pioglitazone therapy would have a synergistic effect on the glucose-lowering effect of metformin through the induction of the drug transporter OCT1. In addition, the enhancement of glucose uptake into cells by GLUT1 and GLUT4 should be considered simultaneously. We therefore compared the glucose-lowering effect of metformin using the Oral Glucose Tolerance Test (OGTT) before and after pioglitazone treatment and determined the m-RNA expression levels of OCT1, GLUT1, and GLUT4 in blood and the glucose-lowering effects of metformin before and after pioglitazone treatment in healthy participants.

Research Design and Methods

Participants

Fifteen healthy male patients (n=15; age, 25 ± 5 years; height, 174.7 ± 7.2 cm; weight, 72.6 ± 8.7 kg; fasting glucose level, 86 ± 10 mg/dl) were recruited for participation in the present study. The exclusion criteria were anemia (hemoglobin <12 g/dl), history of drug abuse, symptomatic coronary heart disease, significant hepatic enzyme elevation (aspartate aminotransferase or alanine aminotransferase >60 IU/L), serum creatinine >1.5 mg/dl, or any of the criteria of metabolic syndrome [16]. Participants consuming >2 alcoholic drinks (at one time) twice weekly, smoking >10 cigarettes per day, or taking any medication were also excluded.

Clinical study procedures

The study protocol was reviewed and approved by the Institutional Review Board of Severance Hospital in the Yonsei University Health System, Seoul, Korea. All procedures were performed in accordance with the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use-Good Clinical Practice guideline. Written, informed consent for participation was obtained from all participants before study enrollment. The participants were asked to maintain normal physical activity at least 5 days before the study began. Dieticians instructed the participants regarding the meal plan designed to maintain a carbohydrate intake of 200 to 250 g/d and the use of a food diary to record food intake for 3 days before admission. The last meal before admission was eaten in the Clinical Trials Center at Severance Hospital. After an overnight fast (more than 14 h), a blood sample was drawn to determine OCT1, GLUT1, and GLUT4 mRNA levels, and a 3-h 75 g OGTT was performed at 10 AM (Day 1). Participants received a 1000-mg oral dose of metformin (Diabex Tab; Daewoong Pharmaceutical Co., Seoul, Korea) at 8 PM. After an overnight fast, a 750-mg dose of metformin was administered at 8 AM on Day 2, followed by the second OGTT at 10 AM. Participants continued taking pioglitazone (Actos Tab; Eli Lilly and Company, Seoul, Korea) 30 mg daily at 8 AM on an outpatient basis from Day 3 to Day 16. They restarted the carbohydrate-controlled diet on Day 16. The participants were admitted to the Clinical Trials Center on Day 18 and released on Day 19. The OGTT tests and metformin administration were performed according to the same schedule used in Day 1 and Day 2.

Blood collection time

The blood samples used to determine OCT and GLUT mRNA levels were collected before the OGTT on Days 1 and 18. For OGTT analysis, blood samples were collected before glucose ingestion and 15, 30, 45, 60, 90, 120, 150, and 180 min after participants ingested 75 g of glucose.

Glucose profile analysis from the OGTT

Metformin lowers glucose production in diabetic patients and exerts the same effect in healthy participants if serum glucose levels are increased by glucose ingestion [8]. The OGTT was conducted four times: before and after metformin treatment prior to pioglitazone administration (Days 1 and 2), and after pioglitazone administration (Days 18 and 19). The maximum glucose level (G_{max}) was determined, and the area under the serum glucose concentration-time curve (AUC_{gluc}) was calculated using the trapezoidal rule. AUC_{gluc60} was defined as the area under the glucose curve from 0 to 60 min after glucose ingestion, during which time the plasma glucose concentration increases. As the purpose of this study was to observe the induction effect of pioglitazone, the administration of pioglitazone was terminated 2 days prior to admission. The glucose-lowering action of metformin was calculated as the differences in G_{max} and AUC_{gluc} values between Days 1/2 and Days 18/19 in each participant (ΔG_{max}, ΔAUC_{gluc60}, and ΔAUC_{gluc}).

OCT1, GLUT1, and GLUT4 mRNA expression levels

The OCT1, GLUT1, and GLUT4 mRNA levels in blood were determined by real-time polymerase chain reaction (PCR). Total RNA was extracted with the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Equal amounts of RNA (500 ng) from each sample were reverse transcribed with an oligo(dT) primer and RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified with human OCT1-specific, GLUT1-specific, and GLUT4-specific TaqMan® gene expression primer-probe sets (Hs00427550_m1, Hs00892681_m1, and Hs00168966_m1, respectively), TaqMan® Gene Expression Master Mix, and the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The following cycling conditions were used: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 50 cycles of 95°C for 15 s followed by 60°C for 1 min. A negative control using water as the template was included in every PCR experiment. Target gene mRNA levels were normalized to that of the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH: 4333764F)

Statistical analysis

Measurements from the same participants before and after pioglitazone treatment were compared using the Wilcoxon signed-rank test. The data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as means ± standard deviation (SD). $P<0.05$ was considered significant.

Results

Glucose-lowering effect of metformin

Healthy volunteers (n=15) underwent an OGTT before and after receiving two doses of metformin on Days 1 and 2 and again on Days 18 and 19 (after a 14-day course of pioglitazone).Baseline serum glucose concentrations (before the first metformin dose) were similar before and after pioglitazone treatment. The glucose-lowering effect of metformin was considerably increased by the 14-day pioglitazone treatment (Figure 1). The ability of metformin to reduce ΔG_{max}, ΔAUC_{gluc60}, and glucose AUC for the entire 180-min test was compared before and after pioglitazone treatment (Table 1). Pioglitazone treatment increased the mean ΔG_{max} by 50.0% (26 mg/dl vs. 39 mg/dl; $P=0.021$) and the mean ΔAUC_{gluc60} by 88.1% (613±508 mg/dl·min vs. 1153 ± 745 mg/dl·min; $P=0.020$). The mean ΔAUC_{gluc} was increased by 266% after pioglitazone treatment (744 ± 1009 mg/dl·min vs. 1981 ± 1525 mg/dl·min; $P=0.008$). The baseline glucose parameter $G_{max\_baseline}$ was decreased insignificantly by 9.5% after pioglitazone treatment (147 ± 21 mg/dl vs. 133 ± 23 mg/dl; $P=0.069$). $AUC_{gluc60\_baseline}$ was decreased by 10.9% after pioglitazone treatment (7367 ± 1194 mg/dl·min vs. 6567 ± 948 g/dl·min; $P=0.017$), and $AUC_{gluc\_baseline}$ was decreased by 8.5% after pioglitazone treatment (18,493 ± 2256 mg/dl·min vs. 16,925 ± 1948 g/dl·min; $P=0.012$).

OCT1, GLUT1, and GLUT4 mRNA expression levels

To characterize the mechanism by which pioglitazone enhanced the glucose-lowering action of metformin, OCT1, GLUT1, and GLUT4 mRNA levels in peripheral blood cells were determined by real-time PCR. To characterize whether the main mechanism of this enhancement is due to enhanced metformin transport activity or

Parameter	Before pioglitazone	After pioglitazone	Ratio [95% CI]	P
$\Delta\,G_{max}$ (mg/dl)	26 ± 11	39 ± 22	1.117 [0.487-1.512]	0.021
ΔAUC_{gluc60} (mg/dl·min)	613 ± 508	1,153 ± 745	1.076 [0.572-2.023]	0.020
ΔAUC_{gluc} (mg/dl·min)	744 ± 1009	1,981 ± 1525	0.881 [0.777-1.609]	0.008
$G_{max\_baseline}$	147 ± 21	133 ± 23	0.899 [0.803-1.006]	0.069
$AUC_{gluc60\_baseline}$	7367 ± 1194	6567 ± 948	0.888 [0.794-0.993]	0.017
$AUC_{gluc\_baseline}$	18493 ± 2256	16925 ± 1948	0.913 [0.837-0.996]	0.012
OCT1 (ΔCts)	−5.63 ± 2.19	−5.48 ± 2.13		0.315
GLUT1 (ΔCts)	−4.41 ± 2.22	−4.62 ± 2.01		0.334

Data were evaluated by Wilcoxon's signed-rank test and expressed as means ± SD. ΔG_{max}, difference in the maximum glucose level before and after metformin treatment; ΔAUC_{gluc60}, difference in the partial glucose AUC (0 to 60 min after ingestion, during which time the glucose concentration increases) before and after metformin treatment; ΔAUC_{gluc}, difference in the total AUC_{gluc} before and after metformin treatment.

Table 1: Glucose profile parameters of the effect of metformin before and after pioglitazone treatment in healthy participants (n=15).
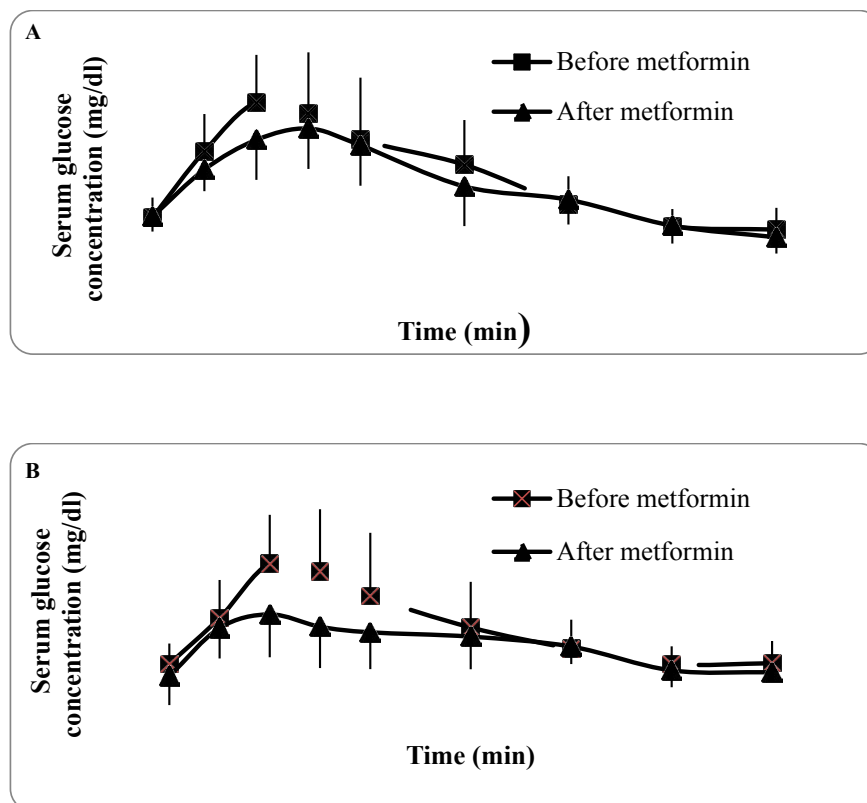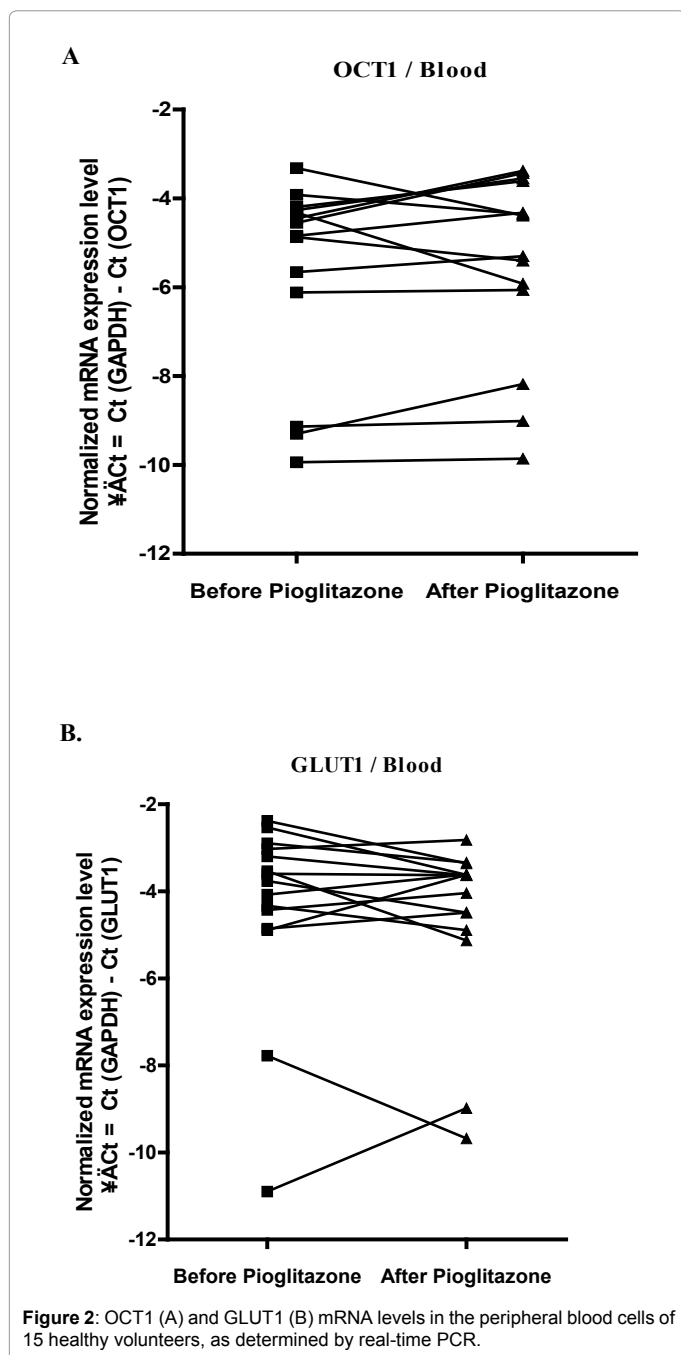

Figure 1: Serum glucose levels were determined by the OGTT before and after metformin administration. (A) Serum glucose profiles before the 14-day administration of pioglitazone. (B) Serum glucose profile after the 14-day administration of pioglitazone. Data are expressed as means ± SD (n=15).

the remaining insulin-sensitizing action of pioglitazone 3 days after stopping pioglitazone treatment, the mRNA levels of OCT1, GLUT1, and GLUT4 were quantified and compared. The amount of mRNA was determined by the delta Ct (ΔCt) method, and the mRNA level of each gene was normalized to that of GAPDH. OCT1 and GLUT1 mRNA levels did not significantly increase after pioglitazone treatment (Figure 2). The ΔCts of OCT1 were −5.63 ± 2.19 and −5.48 ± 2.13 before and after pioglitazone treatment, respectively (P=0.315), and those of GLUT1 were −4.41 ± 2.22 and −4.62 ± 2.01 before and after

pioglitazone treatment, respectively (P=0.334). GLUT4 mRNA was not detected in the peripheral blood cells.

Conclusion

Many studies have demonstrated the in vitro and *in vivo* insulin-mediated induction mechanisms of pioglitazone [17-19]. In the present study, we evaluated the synergistic effect of pioglitazone and metformin by comparing glucose levels in healthy volunteers before and after metformin treatment on Days 1/2 and again on Days 18/19 (after a 14-

Figure 2: OCT1 (A) and GLUT1 (B) mRNA levels in the peripheral blood cells of 15 healthy volunteers, as determined by real-time PCR.

day pioglitazone administration). Our data revealed that pioglitazone increased the glucose-lowering effect of metformin (ΔG_{max}, ΔAUC_{gluc60}, and ΔAUC_{gluc} increased by 50%, 188%, and 266%, respectively) but slightly altered $G_{max\_baseline}$, $AUC_{gluc60\_baseline}$, and $AUC_{gluc\_baseline}$ by 9.5%, 10.9%, and 8.5%, respectively. We demonstrated for the first time that the synergistic effect of pioglitazone on the glucose-lowering effect of metformin lasted at least 3 days after stopping pioglitazone administration in humans.

The pioglitazone-induced effects were assessed 3 days after the last dose of pioglitazone was administered. The half-life of pioglitazone is relatively short [approximately 8.3 h [20]. Although we did not measure pioglitazone blood levels, the direct effect of pioglitazone was expected to be extremely low. For that reason, an indirect mechanism

of pioglitazone enhanced the action of metformin 3 days after the last dose of pioglitazone. The compliance of oral hypoglycemic agents is important to control blood sugar levels in diabetic patients [21]. Our results infer that short periods of noncompliance in the combination therapy of oral hypoglycemic agents including pioglitazone are manageable.

To elucidate the mechanism of the indirect effect of pioglitazone, we measured the mRNA levels of OCT1, GLUT1, and GLUT4 in the peripheral blood before and after pioglitazone administration. OCT1 plays an important role in metformin uptake and activity in the liver; however, the OCT1 mRNA levels in peripheral blood were not increased after pioglitazone administration. This result suggests that the synergistic effect of pioglitazone on metformin was not mediated by metformin transport into hepatocytes through the induction of OCT1 expression in humans. However, we cannot completely rule out the possibility of the induction because pioglitazone and other PPAR agonists increase Oct1 expression levels and function in vitro and *in vivo* [13]. The mRNA expression levels in blood are indirect markers that will not achieve sufficient power to detect changes due to the small number of participants and high variability if the degree of induction was small (i.e., less than 2-fold).

GLUT1 has an important role in basal glucose uptake, and GLUT4 has an important role in insulin-mediated glucose uptake [22]. Pioglitazone administration did not increase GLUT1 mRNA expression, and GLUT4 expression was not detected in this study. The synergistic effect mediated by the induction of glucose transporters could not be proven in this study. However, we can infer that the synergistic effect of pioglitazone is mediated by indirect mechanisms including the insulin-mediated induction of GLUT4 or other factors, and this synergistic effect lasts 3 days after stopping pioglitazone administration in humans.

Our study has some limitations. First, OCT1 and GLUT1 mRNA levels in blood may not reflect hepatic OCT1 and adipose tissue GLUT1 mRNA or protein levels. However, it is unethical to perform liver or muscle biopsies in healthy volunteers. Second, the direct effect of pioglitazone cannot be excluded. The administration of pioglitazone was stopped 3 days before metformin administration to elucidate the induction mechanism of pioglitazone. The baseline glucose parameters were not substantially different. Pioglitazone is extensively metabolized in the liver to five metabolites, two of which (M-III and M-IV) are pharmacologically active and have a potency of approximately 40% to 60% of that of pioglitazone [20]. The half-life of these two metabolites is relatively longer than that of the parent drug (24.2 and 26.2 h for M-III and M-IV, respectively) However, the dosing interval between the last dose of pioglitazone and the second dose of metformin is 72 h, and the potency of the metabolite is approximately half that of the parent drug. We may assume that the direct effect of pioglitazone is not as significant as its indirect effect.

In conclusion, we observed for the first time synergism of the glucose-lowering effect of metformin by pioglitazone administration in healthy volunteers even though pioglitazone treatment was stopped 3 days prior to the second dose of metformin. The synergistic effects of pioglitazone appear to be mediated by indirect effects, although the mechanism by which pioglitazone treatment induced the glucose-lowering effect of metformin was unclear based on our mRNA expression results. It is necessary to investigate the mechanism of the indirect synergistic and lasting effects of PPAR agonists on the targets of metformin including transporters and other pharmacodynamic factors in humans more closely.

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