Synthetic Peroxisome Proliferator-Activated Receptor-γ Agonists Restore Impaired Vasorelaxation via ATP-Sensitive K+ Channels by High Glucose

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ABSTRACT
The present study was designed to examine whether in the human artery, synthetic peroxisome proliferator-activated receptor (PPAR)-γ agonists restore vasorelaxation as well as hyperpolarization via ATP-sensitive K+ channels impaired by the high concentration of d-glucose and whether the restoration may be mediated by the antioxidant capacity of these agents. The isometric force and membrane potential of human omental arteries without endothelium were recorded. The production rate of superoxide was evaluated using a superoxide-generating system with xanthine-xanthine oxidase in the absence of uric acid. Addition of d-glucose (20 mM) but not l-glucose (20 mM) reduced this vasorelaxation and hyperpolarization. Synthetic PPAR-γ agonists (troglitazone and rosiglitazone) and/or an inhibitor of superoxide generation (4,5-dihydroxy-1,3-benzene-disulfonic acid, Tiron), but not a PPAR-α agonist (fenofibrate), restored vasorelaxation and hyperpolarization in response to levcromakalim in arteries treated with d-glucose. Troglitazone and rosiglitazone, but not fenofibrate, decreased the production rate of superoxide without affecting uric acid generation. These findings suggest that synthetic PPAR-γ agonists recover the function of ATP-sensitive K+ channels reduced by the high concentration of glucose in human vascular smooth muscle cells and that the effect of these agonists may be mediated in part by their antioxidant capacity.

ATP-sensitive K+ channels play important roles in physiological and pathophysiological vasodilation (Quayle et al., 1997). In humans as well as animals, diabetes mellitus impairs the activity of ATP-sensitive K+ channels in vascular smooth muscle cells, resulting in decreased vasodilation mediated by these channels (Mayhan and Faraci, 1993; Zimmermann et al., 1997; Miura et al., 2003). Our recent study has demonstrated that in the human artery, high concentrations of glucose reduce the activity of ATP-sensitive K+ channels (Kinoshita et al., 2004). These studies indicate that hyperglycemia as well as diabetes mellitus affects vasodilation mediated by ATP-sensitive K+ channels in human blood vessels. In addition, these pathophysiological conditions appear to produce increased levels of superoxide in the vasculature, leading to inhibition of ATP-sensitive K+ channel activity (Liu and Gutterman, 2002; Kinoshita et al., 2004). Therefore, it is crucial to explore tools by which one can reduce oxidative stress in blood vessels to ameliorate the impaired vascular function produced by glucose intolerance. However, whether drugs available clinically to treat diabetes mellitus restore the activity of K+ channels reduced by high concentrations of glucose has not been evaluated.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily and are involved in energy homeostasis (de la Lastra et al., 2004). Three PPAR

ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; SOD, superoxide dismutase; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); U46619, 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F₂₀; DTPA, diethylenetriaminepentaacetic acid; HEPES-H, 4-(2-hydroxyethyl)pipperazine-1-ethanesulfonic acid.
isoforms have been identified: PPAR-α, PPAR-β/δ, and PPAR-γ, which are implicated in several physiological processes, including the differentiation of adipocytes, regulation of lipoprotein, and lipid metabolism (de la Lastra et al., 2004). Synthetic PPAR-α agonists (fibrates) have been used clinically to reduce triglyceride levels by suppressing the transcription of apolipoprotein C-III, activating lipoprotein lipase, and lowering the concentration of total and very low-density lipoprotein triglyceride (Grundy and Vega, 1987). Clinically available synthetic PPAR-γ agonists, including rosiglitazone, improve insulin-mediated glucose uptake into skeletal muscle without increasing endogenous insulin secretion (de la Lastra et al., 2004; Rangwala and Lazar, 2004). These synthetic PPAR agonists are therapeutic agents for the treatment of metabolic syndromes, including noninsulin-dependent diabetes mellitus. Several studies demonstrated that PPAR-γ agonists possess antioxidant activity through their regulation of the expression of SOD and/or NAD(P)H oxidase (Inoue et al., 2001; Hwang et al., 2005). A PPAR-γ agonist, rosiglitazone, may be capable of scavenging oxygen-derived free radicals through its α-tocopherol-like structure (Cominacini et al., 1997; Stumvoll and Häring, 2002). These results indicate that PPAR agonists produce their superoxide-scavenging capacity in a receptor-independent fashion. Indeed, a recent study on RAW 264.7 macrophages has documented that synthetic PPAR-γ agonists receptor independently inhibit the expression of inducible nitric oxide synthase (Crosby et al., 2005).

Therefore, the present study was designed to examine whether in the human artery, synthetic PPAR-γ agonists restore function of ATP-sensitive K+ channels impaired by the high concentration of glucose, and whether the recovery may be mediated by the antioxidant capacity of these agents.

Materials and Methods

The institutional research committee approved this study, and written informed consent was obtained from each patient enrolled in this study. The part of human greater omentum was obtained from patients scheduled for the elective gastric surgery, and all of patients enrolled [46 patients (male 26, female 20), 45–75 years] were without this study. Tiron, and troglitazone were prepared in dimethyl sulfoxide (1:100, v/v) in the absence (SGRControl) or in the presence (SGRDrug) of the active drug.

Drugs. Levocromakalim was a generous gift from GlaxoSmithKline (Greenford, UK). Allopurinol, DTPA, dimethyl sulfoxide, feno-fibrate, ferriytochrome c (from bovine heart), glibenclamide, HEPES-H, papaverine, rosiglitazone, SOD (from bovine erythrocytes), Tiron, troglitazone, U46619, and xanthine were purchased from Sigma Chemical (St. Louis, MO). Xanthine oxidase (from buttermilk) was purchased from Nacalai Tesque, Kyoto, Japan. Stock solutions of levocromakalim, fenofibrate, glibenclamide, rosiglitazone, Tiron, and troglitazone were prepared in dimethyl sulfoxide (3 × 10−3 M) and allopurinol or other drugs were dissolved in NaOH (1.5 × 10−3 M) and distilled water, respectively.

Statistical Analysis. The data are expressed as means ± S.D. Statistical analysis was performed using repeated measures analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons. The linear regression analysis was performed to evaluate the linearity of the superoxide-scavenging capacity as a function of the concentration of PPAR agonists. Differences were considered to be statistically significant at P < 0.05.

Results

Organ Chamber and Electrophysiological Experiments. During submaximal contraction to U46619 (10−7 M), a selective ATP-sensitive K+ channel opener, levocromakalim (10−8–10−5 M) similarly induced concentration-dependent relaxation in the human omental artery treated with L-glucose (20 mM) as well as in the control artery (Fig. 1). A selective ATP-sensitive K+ channel antagonist, glibenclamide (5 × 10−6 M) completely abolished this vasorelaxation and hyperpolarization (Fig. 1), whereas it did not affect the basal tone of the omental artery. Incubation with D-glucose (20 mM) impaired levocromakalim-induced vasorelaxation and hyperpolarization (Fig. 1). Resting membrane potentials did not differ among the groups studied.
(20 mM) = −40.7 ± 6.8 mV; L-glucose (20 mM) plus glibenclamide (5 × 10⁻⁶ M) = −44.7 ± 6.3 mV; D-glucose (20 mM) = −43.7 ± 6.4 mV).

Synthetic PPAR-γ agonists [troglitazone and rosiglitazone (3 × 10⁻⁶–3 × 10⁻⁵ M)] restored vasorelaxation and hyperpolarization in response to levromakalim in arteries treated with D-glucose (20 mM), whereas a PPAR-α agonist fenofibrate (3 × 10⁻⁶–3 × 10⁻⁵ M) did not alter reduced vasorelaxation and hyperpolarization in these arteries (Fig. 2). Resting membrane potentials did not differ among the groups studied [D-glucose (20 mM) = −44.4 ± 1.1 mV, D-glucose (20 mM) plus fenofibrate (3 × 10⁻⁵ M) = −48.6 ± 5.2 mV, D-glucose (20 mM) plus rosiglitazone (3 × 10⁻⁵ M) = −45.8 ± 5.1 mV, and D-glucose (20 mM) plus troglitazone (3 × 10⁻⁵ M) = −48.2 ± 5.8 mV]. Troglitazone and rosiglitazone (3 × 10⁻⁵ M) did not affect the vasorelaxation produced by levromakalim in arteries incubated with L-glucose (20 mM) (Fig. 3).

An inhibitor of superoxide generation, Tiron (10 mM), restored vasorelaxation in response to levromakalim in omental arteries treated with D-glucose (20 mM), whereas it did not affect vasorelaxation induced by levromakalim in arteries treated with L-glucose (20 mM) (Fig. 4). Tiron (10 mM) restored hyperpolarization in response to levromakalim in the omental arteries treated with D-glucose (20 mM) (Fig. 4). However, addition of troglitazone or rosiglitazone (3 × 10⁻⁵ M) to Tiron did not further augment hyperpolarization in arteries treated with D-glucose (20 mM) (Fig. 4). Resting membrane potentials did not differ among the groups studied [D-glucose (20 mM) = −45.4 ± 5.7 mV, D-glucose (20 mM) plus Tiron (10 mM) = −43.0 ± 4.0 mV, D-glucose (20 mM) plus Tiron (10 mM) in combination with troglitazone (3 × 10⁻⁵ M) = −42.4 ± 5.2 mV, and D-glucose (20 mM) plus Tiron (10 mM) in combination with rosiglitazone (3 × 10⁻⁵ M) = −43.3 ± 5.3 mV].

A xanthine oxidase inhibitor allopurinol (10⁻⁴ M) did not alter vasorelaxation in arteries treated with D-glucose (20 mM) (Fig. 5). The maximal vasorelaxations induced by papaverine (3 × 10⁻⁴ M) did not differ among the groups studied (data not shown).

**Estimation of Superoxide-Scavenging Capacity in a Cell-Free Superoxide-Generating System.** Addition of xanthine oxidase (0.004 U/ml) to the reaction mixture containing ferricytochrome c (5 × 10⁻⁵ M) increased the absorbance at 550 nm linearly at least for the initial 2 min. Removal of xanthine (10⁻⁴ M) from the reaction mixture as well as addition of allopurinol (2 × 10⁻⁴ M) abolished the increase in optical density, indicating that the mixture acts as a superoxide generator. Rosiglitazone concentration dependently reduced superoxide generation (Table 1), whereas at concentrations up to 2 × 10⁻⁴ M, it did not change the production rate of uric acid compared with the control mixture containing dimethyl sulfoxide (1:100, v/v). Similar effects of troglitazone on superoxide generation as well as the production rate of uric acid were observed at concentrations up to its limitation of water solubility (≤3 × 10⁻⁵ M). These results indicate that both glitazones possess superoxide-scavenging capacity without affecting the enzymatic activity of xanthine oxidase. Linearity between the concentration of glitazones and the superoxide-scavenging capacity was confirmed by the linear regression analysis for rosiglitazone, r² = 0.95 – 0.99; for troglitazone, r² = 0.88 – 0.97, respectively; n = 4, P < 0.05). The estimated superoxide-scavenging capacity of each agent at 3 × 10⁻⁵ M was 0.057 ± 0.004 µM/min for rosiglitazone or 0.232 ± 0.179 µM/min for troglitazone, respectively (n = 4). Fenofibrate (up to 10⁻⁴ M) failed to influence the superoxide generation, although this PPAR-α agonist significantly increased the enzymatic activity of xanthine oxidase (19.5 ± 0.5% increase compared with control; P < 0.05).

**Discussion**

In the human omental artery, addition of D-glucose (20 mM) but not the same concentration of L-glucose to normal Krebs’ solution reduced vasorelaxation and hyperpolarization mediated by ATP-sensitive K⁺ channels, suggesting that in the human visceral artery, acute exposure to a high concentration of D-glucose (558 mg/dl) inhibits the activity of ATP-sensitive K⁺ channels in vascular smooth muscle cells.
in an osmolarity-independent fashion. These results with high glucose are consistent with a study on diabetic patients, showing the reduction of vasorelaxation via ATP-sensitive K\textsuperscript{\textsubscript{+}} channels in the coronary arteriole (Miura et al., 2003).

Tiron, which is known to inhibit production of superoxide by chelating metal ions in the catalytic enzymes such as NAD(P)H or xanthine oxidase, completely recovered vasorelaxation and hyperpolarization via ATP-sensitive K\textsuperscript{\textsubscript{+}} channels (O'Neil et al., 2001). Hyperglycemia as well as diabetes mellitus reportedly produces increased levels of superoxide in the human vasculature, leading to the inhibition of ATP-sensitive K\textsuperscript{\textsubscript{+}} channel activity in vascular smooth muscle cells (Kinoshita et al., 2004; Gutterman et al., 2005). Previous studies on diabetic animal models also demonstrated such deleterious effects of superoxide on the function of ATP-sensitive K\textsuperscript{\textsubscript{+}} channels in blood vessels (Liu and Gutterman, 2002; Erdös et al., 2004). Therefore, in humans as well as in animals, increased oxidative stress corresponding with glucose intolerance contributes to the malfunction of ATP-sensitive K\textsuperscript{\textsubscript{+}} channels in vascular smooth muscle cells.

Troglitazone and rosiglitazone, but not fenofibrate, restored vasorelaxation and hyperpolarization induced by levocromakalim in arteries treated with high glucose, suggesting that synthetic PPAR-\gamma, but not PPAR-\alpha, agonists ameliorate malfunction of vascular ATP-sensitive K\textsuperscript{\textsubscript{+}} channels induced by hyperglycemia in humans. To determine the mechanisms of action of synthetic PPAR-\gamma agonists on the activity of ATP-sensitive K\textsuperscript{\textsubscript{+}} channels, we evaluated the effects of these agonists in the presence of Tiron. Addition of neither PPAR-\gamma agonist to Tiron further augmented hyperpolarization in arteries treated with high glucose, indicating that these agents act on the same axis related to the modulation of superoxide levels in vascular smooth muscle cells.

In the present study, we have further estimated the antioxidant capacity of synthetic PPAR agonists using a cell-free superoxide-generating system, in which key enzymes related to the inhibitory effects of high glucose on ATP-sensitive K\textsuperscript{\textsubscript{+}} channel function do not exist [e.g., NAD(P)H oxidase]. Our cell-free superoxide-generating system is a simple tool to evaluate whether synthetic PPAR agonists act as superoxide scavengers by neutralizing the free radical. This is because the system consists of a single catalyst (xanthine oxidase) in the presence of xanthine and oxygen, and the end product of this reaction (uric acid) is easily monitored by spectrometry.

Rosiglitazone and troglitazone decreased the generation rate of superoxide without affecting the enzymatic activity of xanthine oxidase, indicating that these PPAR-\gamma agonists may possess the superoxide-scavenging capacity. However, at the
same concentration ($3 \times 10^{-5}$ M), the relative potency of troglitazone to neutralize superoxide was 4 times that of rosiglitazone. One possible explanation suggested for this finding is that the more potent capacity of troglitazone as a superoxide scavenger is due to a hydrophobic feature of this agent. Troglitazone is a thiazolidinedione with a $\alpha$-tocopherol moiety (Stumvoll et al., 2002), and $\alpha$-tocopherol is the predominant and most active form of vitamin E, which is a

**Fig. 3.** Concentration-response curves to levomakalim in the absence or in the presence of rosiglitazone or troglitazone ($3 \times 10^{-5}$ M) obtained in the human omental artery without endothelium treated with L-glucose (20 mM).

**Fig. 4.** Left, concentration-response curves to levomakalim in the absence or in the presence of Tiron (10 mM) obtained in the human omental artery without endothelium treated with D-glucose or L-glucose. *, difference between rings treated with L-glucose and rings treated with D-glucose, that between rings treated with D-glucose and rings treated with D-glucose in combination with Tiron (10 mM), and that between rings treated with D-glucose and rings treated with L-glucose in combination with Tiron (10 mM) are statistically significant ($P < 0.05$). Right, changes in membrane potential of smooth muscle cells induced by levomakalim ($10^{-5}$ M) in the human omental artery treated with D-glucose. Levomakalim-induced hyperpolarization is similarly recovered by Tiron or Tiron in combination with rosiglitazone or troglitazone, respectively (*, $P < 0.05$).

**Fig. 5.** Concentration-response curves to levomakalim in the absence or in the presence of allopurinol ($10^{-4}$ M), obtained in the human omental artery without endothelium treated with D-glucose.
major antioxidant in lipid phases (Traber and Sies, 1996). Therefore, it is also likely that the α-tocopherol-like structure allows troglitazone to easily access the site responsible for superoxide generation and/or reaction. However, it is still unclear how these glitazones carry the common antioxidant capacity because rosiglitazone does not have the α-tocopherol moiety (Stumvoll et al., 2002). In the current study, rosiglitazone and troglitazone similarly restored vasorelaxation moiety (Stumvoll et al., 2002). In the current study, rosiglitazone does not have the α-tocopherol moiety (Stumvoll et al., 2002). In the current study, rosiglitazone and troglitazone similarly restored vasorelaxation and hyperpolarization via ATP-sensitive K+ channels in arteries treated with high glucose even though their antioxidant capacities seem to be different. The antioxidant capacity of rosiglitazone may be enough to overcome the adverse effects of superoxide provoked by glucose intolerance in the human artery.

The amount of superoxide needed to access the redox state of responsible cell structures remains to be established in the present study. The concentration of superoxide yielded from our cell-free generating system (0.004 U/ml xanthine oxidase) is within the range of lethal concentrations for living cells as we have already demonstrated (Azma et al., 1999). Although several percentages of lethal amounts of superoxide generated from our free radical generator were neutralized by glitazones in the current study, our superoxide-scavenging assay may not provide conclusive explanations for the potential mechanism of action of glitazones on superoxide. Further studies are required to resolve this issue regarding the superoxide-scavenging capacity of glitazones.

The effect of fenofibrate to enhance oxidative stress has been reported (Teissier et al., 2004), although in the present study, it did not alter vasorelaxation and hyperpolarization in response to levocromakalim in arteries treated with high glucose. This dissociation may be caused by experimental conditions.

Our recent study has suggested that the increased production of superoxide seen in arteries treated with high glucose is mediated by NAD(P)H oxidase (Kinosita et al., 2004). In contrast, xanthine oxidase, which is a component of our cell-free superoxide-generating system, does not seem to be responsible for the superoxide generation in human vascular smooth muscle cells (Moriwaki et al., 1993). Indeed, our negative results with allopurinol indicate that xanthine oxidase is not involved in the inhibition of the ATP-sensitive K+ channel function provoked by high glucose. We believe that this lack of relation of our superoxide generator (xanthine-xanthine oxidase) to ATP-sensitive K+ channel function should be rather an advantage because the interaction between xanthine oxidase and PPAR-γ agonists, unlike that between NAD(P)H oxidase and PPAR-γ agonists, has never been demonstrated (Inoue et al., 2001; Bagi et al., 2004; Hwang et al., 2005). In the current study, glitazones did not affect the enzymatic activity of xanthine oxidase, supporting the conclusion that the antioxidant effects of synthetic PPAR-γ agonists may play a role in part in the recovery of ATP-sensitive K+ channel function impaired by high glucose and that in the presence of endothelial xanthine oxidase, the possible superoxide-scavenging capacity of glitazones may be interfered with by the superoxide produced by this enzyme.

The peak plasma concentrations of rosiglitazone and fenofibrate used in clinical practice have been reported as ~2 × 10^{-6} or 3 × 10^{-5} M, respectively (Miller and Spence, 1998; Wagstaff and Goa, 2002). Therefore, the present results regarding the effects of synthetic PPAR agonists on human vascular function via ATP-sensitive K+ channels suggest that in clinical situations, PPAR-γ, but not PPAR-α, agonists may restore the ATP-sensitive K+ channel function reduced by hyperglycemia.

This is the first study demonstrating that in the human artery synthetic PPAR-γ agonists restore function of ATP-sensitive K+ channels impaired by a high concentration of glucose. A recent study on rats has demonstrated that rosiglitazone restores the gastric damage induced by ischemia and reperfusion, suggesting the counteracting effect of PPAR-γ agonists on oxidative stress in the digestive apparatus (Villegas et al., 2004). It is likely that in humans, ATP-sensitive K+ channels regulate pathophysiological as well as pharmacological vasodilation, whereas these channels do not contribute resting membrane potentials, even in the condition with glucose intolerance (Quayle et al., 1997; Zimmermann et al., 1997; Kinoshita et al., 2004). Indeed, acidosis corresponding with ischemia reportedly activates ATP-sensitive K+ channels, resulting in visceral arterial dilation, which indicates important roles of these channels in the regulation of human visceral circulation (Wang et al., 2003). It is not clinically rare to administer vasodilators such as nicorandil, which act via ATP-sensitive K+ channels, to patients with insulin tolerance (Mannhold, 2004). Together with our results, it can be concluded that synthetic PPAR-γ agonists may ameliorate the malfunction of ATP-sensitive K+ channels induced by the conditions with increased oxidative stress, including acute glucose intolerance.

References


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