Metformin normalizes endothelial function by suppressing vasoconstrictor prostanoids in mesenteric arteries from OLETF rats, a model of type 2 diabetes

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Matsumoto T, Noguchi E, Ishida K, Kobayashi T, Yamada N, Kamata K. Metformin normalizes endothelial function by suppressing vasoconstrictor prostanoids in mesenteric arteries from OLETF rats, a model of type 2 diabetes. Am J Physiol Heart Circ Physiol 295: H1165–H1176, 2008. First published July 18, 2008; doi:10.1152/ajpheart.00486.2008.—We previously reported that in mesenteric arteries from aged Otsuka Long-Evans Tokushima fatty (OLETF) rats (a type 2 diabetes model) endothelium-derived hyperpolarizing factor (EDHF)-type relaxation is impaired while endothelium-derived contracting factor (EDCF)-mediated contraction is enhanced (Matsumoto T, Kakami M, Noguchi E, Kobayashi T, Kamata K. Am J Physiol Heart Circ Physiol 293: H1480–H1490, 2007). Here we investigated whether acute and/or chronic treatment with metformin might improve this imbalance between the effects of the above endothelium-derived factors in mesenteric arteries isolated from OLETF rats. In acute studies on OLETF mesenteric arteries, ACh-induced relaxation was impaired and the relaxation became weaker at high ACh concentrations. Both metformin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside [AICAR, an AMP-activated protein kinase (AMPK) activator that is also activated by metformin] 1 diminished the tendency for the relaxation to reverse at high ACh concentrations and 2) suppressed both ACh-induced EDCF-mediated contraction and ACh-stimulated production of prostanoids (thromboxane A2 and PGF2α). In studies on OLETF arteries from chronically treated animals, metformin treatment (300 mg·kg−1·day−1 for 4 wk) 1) improved ACh-induced nitric oxide- or EDHF-mediated relaxation and cyclooxygenase (COX)-mediated contraction, 2) reduced EDCF-mediated contraction, 3) suppressed production of prostanoids, and 4) reduced superoxide generation. Metformin did not alter the protein expressions of endothelial nitric oxide synthase (eNOS), phospho-eNOS (Ser1177), or COX-1, but it increased COX-2 protein. These results suggest that metformin improves endothelial functions in OLETF mesenteric arteries by suppressing vasoconstrictor prostanoids and by reducing oxidative stress. Our data suggest that within the timescale studied here, metformin improves endothelial function through this direct mechanism, rather than by improving metabolic abnormalities.

cyclooxygenase; endothelium-derived contracting factor; endothelium-derived hyperpolarizing factor

TYPE 2 DIABETES MELLITUS (T2DM) and associated components of the dysmetabolic syndrome are already leading causes of morbidity and mortality in societies around the world, yet recent estimates predict a doubling in T2DM prevalence by 2025 (66). T2DM is associated with a markedly increased incidence of cardiovascular diseases. However, the exact relationships among T2DM, obesity, and cardiovascular disease are not completely understood and indeed are the subject of some dispute. T2DM is often part of an array of complex abnormalities referred to as “metabolic syndrome,” which is frequently accompanied by an elevated blood pressure (52).

Vascular tone is controlled by endothelium-derived factors such as relaxing factors [EDRFs; in particular, nitric oxide (NO) and hyperpolarizing factors (EDHFs)] and contracting factors (EDCFs) (7, 12, 49, 60). Changes in these factors may be the cause of changes in resting blood pressure. Several lines of evidence suggest that endothelial dysfunction could play a key role in the development of both macro- and microangiopathy in diabetes patients and in animal models of diabetes (10, 32, 49). An accumulating body of evidence indicates that endothelium-dependent relaxation is impaired in several blood vessels in T2DM [both in animal models and in patients (10, 17, 27, 39)] as well as in type 1 diabetes (10, 16, 19, 26, 31, 36, 38, 42). Furthermore, several reports have suggested that a decreased production of EDRFs and/or defects in EDRF signaling may underlie the impairment of endothelium-dependent relaxation seen in type 2 diabetic vessels (10, 34, 35). Moreover, this impairment of relaxation may be attributable not only to reduced amounts of EDRF and/or EDHF but also to increased amounts of EDCF, leading to diabetic vasculopathy (10, 32, 60). Although the genesis of these alterations remains poorly understood, the contributions of a number of potentially important variables have been examined. These include hyperglycemia (63), altered antioxidant balance (56), alterations in the circulating lipid profile [including elevation in free fatty acids (FFA)] (53), and insulin resistance (1). It still remains questionable, however, whether and to what extent changes in such factors might contribute to vasculopathy in T2DM.

Metformin, a biguanide derivative (dimethylbiguanide), is one of the most commonly used drugs for the treatment of T2DM (59). Metformin exerts an antihyperglycemic effect, with minimal risk of hypoglycemia, and it has recently been used against T2DM, with a 31% reduction in incidence (25). Moreover, in overweight T2DM patients, metformin use is associated with decreases in macrovascular morbidity and mortality, effects that appear to be independent of the improvement in glyemic control (59). Hence, metformin may have a specific protective vascular effect beyond its antihyperglycemic-
mic action. Metformin not only lowers blood glucose concentration but also inhibits adipose tissue lipolysis, reduces circulating levels of FFA, reduces the rate of formation of advanced glycation end products, and improves insulin sensitivity (23). In addition, metformin improves lipid profiles and lowers blood pressure in both patients and animals with impaired glucose tolerance and T2DM (23, 61). Despite the long history and success of metformin as a treatment for T2DM, however, its molecular mechanisms are still poorly defined, although admittedly several reports (57, 67) have provided evidence that metformin activates AMP-activated protein kinase (AMPK), a major cellular regulator of lipid and glucose metabolism (57).

Otsuka Long-Evans Tokushima fatty (OLETF) rats are characterized by an early increase in serum insulin and also by late-onset hyperglycemia, mild obesity, and mild T2DM (21), and there are several reports of abnormalities of vascular function in this diabetic model (18, 33–35). Moreover, we recently demonstrated (30) that endothelial dysfunction is present in the mesenteric arteries of aged OLETF rats, 2) that this may result from an imbalance between endothelium-derived factors (reduced EDRF signaling and increased EDCF signaling), and 3) that the mechanisms underlying this abnormality may involve increments in both cyclooxygenase (COX)-1 and COX-2 activities. We therefore proposed important roles for COX-derived constrictor PGs in the altered regulation of mesenteric arterial responsiveness seen in OLETF rats (30). Although there is an accumulating body of evidence to show that metformin has beneficial effects on diabetic complications in both patients and animal models, no study has yet investigated the acute and chronic effects of metformin on the balance between the effects of EDRFs and EDCFs in the type 2 diabetic mesenteric artery. In this study, we carried out just such an investigation, and further tried to determine the underlying mechanism, using mesenteric arteries isolated from OLETF rats.

MATERIALS AND METHODS

Reagents. 1-[2-Chlorophenyl]diphenylmethyl]-1H-pyrazole (TRAM-34), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), apamin, phenylephrine (PE), indomethacin, N03,nitro-L-arginine (L-NNA), nitro blue tetrazolium (NBT), and monoclonal β-actin antibody were all purchased from Sigma Chemical (St. Louis, MO), while acetylcholine chloride (ACh) was purchased from Daiichi-sankyo Pharmaceuticals (Tokyo, Japan). Antibodies against COX-1 and COX-2 were from Cayman Chemical (Ann Arbor, MI). Drugs were dissolved in saline, except for TRAM-34 (dissolved in DMSO) and indomethacin (dissolved first in a small amount of 0.1 M Na2CO3 solution and then made up to the final volume with distilled water). Horseradish peroxidase (HRP)-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI), while the antibody against endothelial nitric oxide synthase (eNOS) was from BD Biosciences (San Jose, CA). Phospho-eNOS (P-eNOS) (Ser1177) antibody was purchased from Cell Signaling Technology (Danvers, MA).

Animals and experimental design. Five-week-old male rats [OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats, a genetic control for OLETF] were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). Food and water were given ad libitum in a controlled environment (room temperature 21–22°C, room humidity 50 ± 5%) until the rats were 36–42 wk old. For the chronic study, some OLETF and LETO rats were chronically given metformin (300 mg·kg⁻¹·day⁻¹ po) for 4 wk starting at 36–42 wk of age. Thus we studied four groups: untreated LETO and OLETF groups and metformin-treated LETO and OLETF groups. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Measurement of blood glucose, cholesterol, triglyceride, insulin, leptin, nonesterified fatty acid, and blood pressure. Plasma parameters and systemic blood pressure were measured as described previously (31, 36–38, 40, 41). Briefly, plasma glucose, cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, and serum nonesterified fatty acid (NEFA) levels were each determined by the use of a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (EIA) (Shibayagi, Shibukawa, Japan). Plasma leptin was determined by ELISA (Mornina Institute of Biological Science, Yokohama, Japan). The plasma antioxidant level was determined with the use of a commercially available kit (Cayman Chemical). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its blood pressure was measured by the tail-cuff method with a blood pressure analyzer (BP-98A, Softron, Tokyo, Japan).

Measurement of isometric force. Vascular isometric force was recorded as we described previously (30, 31, 34, 36, 40, 41). At 40–46 wk of age, rats were anesthetized with diethyl ether and then euthanized by decapitation. The superior mesenteric artery was rapidly removed and immersed in oxygenated modified Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO3, 1.2 CaCl2, 1.2 NaH2PO4, 1.2 MgSO4, and 11.0 dextrose. The artery was carefully cleaned of all fat and connective tissue, and ring segments 2 mm in length were suspended by a pair of stainless steel pins in a well-oxygenated (95% O2-5% CO2) bath containing 10 ml of KHS at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was loaded and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T, Nihon Kohden, Tokyo, Japan).

For the relaxation studies, mesenteric rings were precontracted with an equally effective concentration of PE (0.1–3 μM) (i.e., so that the tension developed in response to PE was similar among all groups). There was no significant difference in the response to PE among the LETO (n = 51), OLETF (n = 51), metformin-treated LETO (n = 32), and metformin-treated OLETF (n = 30) groups (1.60 ± 0.03, 1.68 ± 0.03, 1.63 ± 0.04, and 1.68 ± 0.04 g, respectively). When the PE-induced contraction had reached a plateau level, ACh (1 nM–10 μM) was added in a cumulative manner. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. To investigate the acute effects of drugs [3 mM metformin, 100 μM AICAR (an AMPK activator), or 10 μM indomethacin (to investigate the influence of COX metabolites)] on ACh-induced endothelium-dependent relaxation, rings were incubated with a given drug for 30 min before administration of PE. In the chronic study, to investigate the influences of the various factors that might constitute EDRF in the present preparations, we examined ACh-induced relaxation in the absence or presence of various inhibitors, as follows: 1) 10 μM indomethacin plus 100 μM L-NNA (to investigate EDHF-type relaxation), 2) 10 μM indomethacin plus 10 μM TRAM-34 [specific inhibitor of the intermediate-conductance Ca2⁺-activated K⁺ (KCa) channel] plus 100 nM apamin [specific inhibitor of the small-conductance KCa (SKCa) channel] (to investigate NO-mediated relaxation), and 3) 100 μM L-NNA plus 10 μM TRAM-34 plus 100 nM apamin (to investigate COX-mediated relaxation). Rings were incubated with the appropriate inhibitor(s) for 30 min before administration of PE.
For the contraction studies, mesenteric rings were first contracted with 80 mM K⁺, these responses being taken as 100%. There was no significant difference in the response to 80 mM K⁺ among the LETO (n = 32), OLETF (n = 32), metformin-treated LETO (n = 16), and metformin-treated OLETF (n = 16) groups (1.63 ± 0.02, 1.69 ± 0.03, 1.65 ± 0.03, and 1.63 ± 0.04 g, respectively). After washing and equilibration for 1 h, PE (1 mM -10 μM) was added cumulatively to the bath until a maximal response was achieved. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. Neither the maximal response (% of 80 mM K⁺) nor the EC50 value (0.77 nM) for OLETF (n = 8), metformin-treated LETO (n = 8), and metformin-treated OLETF (n = 8) groups. To investigate the EDCF-mediated response, mesenteric rings were treated with 100 μM L-NNa for 30 min. After this incubation period, ACh (10 nM – 10 μM) was applied.

Release of prostaglandins. Prostanoid release was measured as we described previously (30). To allow us to measure the release of prostanoids, mesenteric arteries from metformin-treated and untreated LETO and OLETF groups were cut into transverse rings 4 mm in length. These were placed for 30 min in siliconized tubes containing 1.0 ml of KHS in the presence of 100 μM L-NNa at 37°C, and then 10 μM ACh was applied for 15 min. To investigate the effect of 3 mM metformin or 100 μM AICAR on ACh-stimulated prostanoid production in mesenteric arteries from LETO and OLETF groups, mesenteric rings were left for 30 min in the presence of either 100 μM L-NNa plus 3 mM metformin or 100 μM L-NNa plus 100 μM AICAR, and then 10 μM ACh was applied for 15 min. Next, after the mesenteric rings had been removed, the tubes were freezed-clamped in liquid nitrogen and stored at −80°C for later analysis. The prostaglandins were measured with a commercially available ELIA kit (Cayman Chemical). Two-time diluted 50-μl samples were used for measurements of PGE2, thromboxane (TxB2) (a stable metabolite of TxA2), and PGF2α, whereas one hundred-time diluted 50-μl samples were used for measurements of 6-keto PGF1α (stable metabolite of prostacyclin). The various assays were performed as described in the manufacturer’s procedure booklet. The amounts of prostaglandins released are expressed as picograms per milligram of wet weight of mesenteric artery.

Measurement of nitrite and nitrate. The concentration of nitrite (NO3⁻) plus nitrate (NO2⁻) in the plasma (plasma NOx) was assayed by the method described by us elsewhere (30, 38). For the determination of plasma NOx, 0.3 ml of each plasma sample 0.3 ml of 100% methanol was added and the sample was then centrifuged at 5,000 g for 10 min at 4°C. Briefly, the NOx in the plasma was separated by means of a reverse-phase separation column packed with polystyrene polyether (NO-PAK, 4.6 × 50 mm, Eicom, Kyoto, Japan), after which NO3⁻ was reduced to NO2⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). The NO2⁻ was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven set at 35°C. The absorbance of the product dye at 540 nm was measured with a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl/NH4Cl and 0.5 g/4 ml EDTA. The Griess reagent, which was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-naphthyl-ethylenediamine, was delivered at a rate of 0.1 ml/min. The concentrations of NO3⁻ and NO2⁻ and the reliability of the reduction column were examined in each experiment.

Western blotting. The protein levels of COXs, eNOS, and P-eNOS (Ser1177) were quantified with immunoblotting procedures essentially as described previously (40). Mesenteric arterial tissues (2 pooled vessels per group) were homogenized in ice-cold lysis buffer (containing 50 mM Tris·HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS containing protease and phosphatase inhibitor cocktails (Complete Protease Inhibitor Cocktail and PhosSTOP, Roche Diagnostics, Indianapolis, IN). The lysate was cleared by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was collected, and the proteins were solubilized in Laemmli buffer containing mercaptoethanol. Protein concentrations were determined by means of a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). Samples (20 μg/lane) were resolved by electrophoresis on 7.5% or 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after the residual protein sites on the membrane were blocked with ImmunoBlock (Dainippon-Pharm, Osaka, Japan), the membrane was incubated with anti-COX-1 (70 kDa; 1:500), anti-COX-2 (72 kDa; 1:500), anti-eNOS (140 kDa; 1:1,000), or anti-P-eNOS (Ser1177) (140 kDa; 1:1,000) in blocking solution. HRP-conjugated anti-mouse or anti-rabbit antibody was used at a 1:10,000 dilution in TMB-Potio followed by detection with SuperSignal (Pierce). For normalization, we used β-actin as a housekeeping protein. The β-actin protein levels were determined after stripping the membrane and probing with β-actin monoclonal primary antibody (42 kDa; 1:5,000), with HRP-conjugated anti-mouse IgG as the secondary antibody. For analysis of P-eNOS expression, detection of phosphoprotein was followed by membrane stripping and by detection of total eNOS expression and then of β-actin (see above). The optical densities of the bands on the film were quantified by densitometry, with correction for the optical density of the corresponding β-actin band.

Quantification of superoxide anion by measurement of the amount of NBT reduced. Mesenteric artery rings were incubated with NBT to allow the superoxide generated by the tissue to reduce the NBT to blue formazan. The details of the assay have been published previously (35, 37, 38). Briefly, mesenteric arteries from metformin-treated and untreated LETO and OLETF groups were cut into transverse rings 4 mm in length, and these were placed for 120 min at 37°C in 1 ml of KHS containing NBT (100 μM) in the presence of ACh (10 μM). The NBT reduction was stopped by the addition of 0.5 N HCl (1 ml). After this incubation, the rings were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg/l diethylamine pentaacetic acid. The mixture was centrifuged at 16,000 g for 30 min, and the resultant pellet was resuspended in 250 μl of pyridine at 80°C for 60 min to extract formazan. The mixture was then subjected to a second centrifugation at 10,000 g for 10 min. The absorbance of the formazan was determined spectrophotometrically at 540 nm. The amount of NBT reduced (= quantity of formazan) was calculated as follows: amount of NBT reduced = A × V/(T × Wt × e × l), where A is the absorbance, V is the volume of pyridine, T is the time for which the rings were incubated with NBT, Wt is the blotted wet weight of the aortic rings, e is the extinction coefficient (0.71 mmol⁻¹·l⁻¹·mm⁻¹), and l is the length of the light path. The results are reported in picomoles per minute per milligram of Wt.

Statistical analysis. Data are expressed as means ± SE. Each relaxation response is expressed as a percentage of the contraction induced by PE. Contractile responses are expressed as a percentage of the response to 80 mM KCl. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance, a probability level of P < 0.05 being considered significant. Statistical comparisons between concentration-response curves were made with a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc (P < 0.05 again being considered significant).

RESULTS

General parameters. As shown in Table 1, at the time of the experiment all OLETF rats (nonfasted) exhibited hyperglycemia, their blood glucose concentrations being significantly
higher than those of the age-matched nondiabetic control LETO rats (also nonfasted). The body weight of the OLETF rats was greater than that of the LETO rats. Plasma triglyceride, insulin, leptin, and NEFA levels were all significantly higher in OLETF rats than in LETO rats, while HDL concentrations were similar between the two groups (Table 1). The total cholesterol concentration tended to be higher in OLETF rats than in LETO rats (although statistical significance was not reached) (Table 1). The systolic blood pressure of OLETF rats was higher than that of LETO rats, while heart rate was similar between the two groups. Treatment with metformin did not alter plasma total cholesterol, triglyceride, HDL, insulin, leptin, NEFA, or heart rate in OLETF rats, but it significantly lowered body weight, systolic blood pressure, and plasma glucose (vs. nontreated OLETF rats). In LETO rats, treatment with metformin significantly lowered body weight and plasma leptin (vs. nontreated LETO rats).

**Effects of acute metformin treatment on endothelium-dependent relaxation in OLETF rats.** We previously demonstrated (30) that mesenteric arteries from aged OLETF rats exhibit endothelial dysfunction and that this results from an imbalance of endothelium-derived factors (reduced EDRF signaling and increased EDCF signaling). As shown in Fig. 1A, ACh induced a concentration-dependent relaxation (with the maximum response being at 100–300 nM, and responses then being progressively weaker up to 10 μM), and this relaxation was significantly weaker in rings from OLETF rats than in those from LETO rats. For a direct evaluation of the acute actions of metformin on this endothelium-dependent relaxation, mesenteric rings were preincubated with 3 mM metformin for 30 min. This treatment had no significant effect on ACh-induced relaxation in the LETO group (Fig. 1B). In the OLETF group, however, metformin (and also indomethacin) greatly reduced or eliminated the tendency for the relaxation to weaken (or reverse) as the concentration of ACh was increased (0.3–10 μM) (Fig. 1C). Similarly, in OLETF rings treated with AICAR (100 μM; an activator of AMPK, which is also activated by metformin (9, 57, 67)) the relaxation did not weaken at higher concentrations of ACh. However, AICAR significantly reduced the relaxation evoked by ACh (at ACh concentrations of 0.1 and 0.3 μM) in mesenteric rings obtained from LETO rats (Fig. 1B). These results indicate that products of COX metabolism make a prominent contribution to the decrease in endothelium-dependent relaxation that is observed in these preparations at higher ACh concentrations, and they suggest that metformin, like indomethacin, acutely suppresses COX metabolism.

**Effects of acute metformin treatment on endothelium-dependent contraction and ACh-stimulated prostanoid release in OLETF rats.** As described above (Fig. 1), at higher concentrations of ACh (i.e., 1–10 μM), a reduced ACh-induced relax-

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**Table 1. Values of various parameters in metformin-treated and untreated LETO and OLETF rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LETO (5)</th>
<th>Metformin-Treated LETO (5)</th>
<th>OLETF (5)</th>
<th>Metformin-Treated OLETF (5)</th>
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<tr>
<td>Body wt, g</td>
<td>531.6±8.5 (15)</td>
<td>465.6±8.8 (15)†</td>
<td>739.6±18.2 (15)†</td>
<td>660.5±16.2 (15)§</td>
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<td>Glucose, mg/dl</td>
<td>138.2±7.1 (15)</td>
<td>152.3±8.7 (15)</td>
<td>250.8±16.3 (15)†</td>
<td>191.2±9.6 (15)§</td>
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<tr>
<td>Cholesterol, mg/dl</td>
<td>115.4±4.3 (15)</td>
<td>116.6±6.5 (15)</td>
<td>125.9±4.0 (15)</td>
<td>120.8±5.8 (15)</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>71.6±2.7 (15)</td>
<td>65.6±1.1 (15)</td>
<td>315.2±29.8 (15)†</td>
<td>274.6±37.6 (15)†</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>72.5±4.4 (15)</td>
<td>77.2±3.8 (15)</td>
<td>65.8±5.9 (15)</td>
<td>73.1±3.5 (15)</td>
</tr>
<tr>
<td>Insulin, mg/ml</td>
<td>2.4±0.3 (15)</td>
<td>3.2±0.3 (15)</td>
<td>5.8±1.3 (15)*</td>
<td>5.0±0.5 (15)†</td>
</tr>
<tr>
<td>Leptin, mg/ml</td>
<td>9.6±0.7 (12)</td>
<td>12.5±0.5 (12)†</td>
<td>21.7±2.2 (12)†</td>
<td>18.6±1.4 (12)†</td>
</tr>
<tr>
<td>NEFA, meq/l</td>
<td>0.28±0.01 (12)</td>
<td>0.32±0.03 (9)</td>
<td>0.46±0.04 (12)†</td>
<td>0.42±0.03 (12)†</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117.0±2.4 (15)</td>
<td>114.1±2.5 (15)</td>
<td>141.3±2.0 (15)†</td>
<td>135.6±1.9 (15)†</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>403.5±10.4 (15)</td>
<td>420.2±13.9 (15)</td>
<td>423.3±11.4 (15)</td>
<td>406.6±7.9 (15)</td>
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</table>

Values are means ± SE for no. of determinations shown in parentheses. OLETF, Otsuka Long-Evans Tokushima fatty; LETO, Long-Evans Tokushima Otsuka; HDL, high-density lipoprotein; NEFA, nonesterified fatty acid. *P < 0.05, †P < 0.001 vs. LETO; ‡P < 0.05, §P < 0.01 vs. OLETF.
ation was observed, with the relaxation being more nearly abolished in rings from OLETF rats than in those from LETO rats. To investigate the acute effects of metformin on the contractile component of these ACh-induced responses, we added ACh (10 nM–10 μM) cumulatively to rings in the presence of L-NNA (100 μM) as reported previously (30). As shown in Fig. 2A, under these conditions ACh-induced contraction was observed at higher ACh concentrations (i.e., 0.3–10 μM) in rings from both groups. This response was significantly greater in mesenteric arteries from OLETF rats than in those from LETO rats (Fig. 2A). Metformin (3 mM) had no significant effect on this ACh-induced contraction in the LETO group (Fig. 2B). However, the same treatment had a significant suppressive effect on this contraction in the OLETF group (Fig. 2C). Moreover, the ACh-induced contraction was completely blocked by AICAR (100 μM) treatment in both LETO and OLETF groups (Fig. 2B and C).

Since we (30) and others (12, 54, 60) have suggested that vasoconstrictor prostanoids make a prominent contribution to EDCF-mediated responses, we examined the effects of metformin and AICAR on ACh-stimulated protonoid release in mesenteric arteries from LETO and OLETF rats (Fig. 3). The ACh-induced release of TxB2 (stable metabolite of TxA2) and that of PGE2 were significantly greater in rings from OLETF rats than in those from LETO rats. In OLETF rats, both metformin (3 mM) and AICAR (100 μM) significantly reduced each ACh-induced release (TxB2 and PGE2). In LETO rats, AICAR (100 μM) significantly reduced the ACh-induced release of TxB2, but not that of PGE2, while metformin had no significant effect on the ACh-induced release of either prostanoïd. These results are consistent with metformin suppressing EDCF-mediated responses by inhibiting the release of vasoconstrictor prostanoids.

Effects of chronic metformin treatment on endothelial dysfunction in OLETF rats. Having found that metformin has acute effects on endothelial function (Figs. 1–3), we next evaluated whether chronic metformin treatment might improve endothelial dysfunction in OLETF rats. To this end, OLETF rats and LETO rats (36–42 wk old) were treated daily for 4 wk with or without metformin (300 mg·kg⁻¹·day⁻¹ p.o.), and then the following experiments were conducted on isolated mesenteric artery rings.

When the PE-induced contraction had reached a plateau, ACh (1 nM–10 μM) was added cumulatively (Fig. 4). In endothelium-intact preparations of mesenteric artery rings from metformin-treated and untreated LETO and OLETF rats, ACh induced a concentration-dependent relaxation (with the maximum response being at 100–300 nM and responses then

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**Fig. 2.** Endothelium-dependent contractions in mesenteric arteries obtained from LETO and OLETF rats. A: concentration-response curves for ACh-induced contractions of isolated rings of mesenteric arteries in the presence of 100 μM Nω-nitro-L-arginine (l-NNA). B and C: effects of 3 mM metformin or 100 μM AICAR on ACh-induced contraction in the presence of 100 μM l-NNA in mesenteric arteries isolated from LETO (B) and OLETF (C) rats. Details are given in MATERIALS AND METHODS. Data are means ± SE. Number of determinations is shown in parentheses. For comparison, the curves obtained for ACh-induced contraction in the presence of 100 μM l-NNA in A are shown again in B and C. *P < 0.05 vs. LETO group; †P < 0.05, ‡P < 0.01, §§P < 0.001 vs. OLETF group.

**Fig. 3.** Effects of 3 mM metformin or 100 μM AICAR on release of prostanoids [thromboxane (Tx)B2 (stable metabolite of TxA2), A; PGE2, B] evoked by 10 μM ACh in mesenteric artery rings isolated from LETO and OLETF rats. Details are given in MATERIALS AND METHODS. Number of determinations is shown in parentheses. **P < 0.01 vs. control LETO group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. control OLETF group.
being progressively weaker up to 10 μM (Fig. 4A). Overall, this relaxation was significantly weaker in rings from OLETF rats (Fig. 4A). In OLETF rats, but not in LETO rats, metformin treatment significantly improved this ACh-induced relaxation (Fig. 4A).

To investigate which endothelium-derived factors might be improved in mesenteric arteries from metformin-treated OLETF rats, we examined ACh-induced relaxation in the presence of various inhibitors (Fig. 4, B–D–). To investigate the component of the ACh-induced endothelium-dependent relaxation that is mediated by EDHF, we performed a series of experiments in which ACh was added cumulatively to rings precontracted by PE in the presence of 100 μM L-NNA plus 10 μM indomethacin (TRAM-34; C). This EDHF-type relaxation was significantly weaker in rings from OLETF rats than in those from LETO rats (Fig. 4C). In OLETF rats, but not in LETO rats, metformin treatment significantly improved the ACh-induced EDHF-type relaxation (Fig. 4C).

Next, to assess the possible effects of COX metabolites on endothelium-dependent relaxation, mesenteric rings were preincubated with 100 μM L-NNA, 100 nM apamin, and 10 μM TRAM-34 for 30 min. After this treatment, tension developed as the concentration of ACh was increased (0.3–10 μM) in all groups (Fig. 4D), and this contraction was significantly greater in rings from OLETF rats than in those from LETO rats (Fig. 4D). In OLETF rats, but not in LETO rats, metformin treatment significantly decreased this contraction (Fig. 4D). This result indicates that in these preparations products of COX metabolism make a prominent contribution to the weakening of the endothelium-dependent relaxation observed at higher ACh concentrations. Indeed, EDCF-mediated contractions (i.e., the responses induced by ACh in the presence of L-NNA) were significantly reduced by metformin treatment in mesenteric arteries from both LETO and OLETF rats (Fig. 4E).

Effects of chronic metformin treatment on ACh-induced release of prostanoids in OLETF rats. In view of the published evidence (supported by the present findings) that the overproduction of prostanoids contributes to endothelial dysfunction in OLETF rats, we investigated the effect of metformin on ACh-induced prostanoid release. We found that metformin treatment significantly reduced ACh-induced prostanoid release in OLETF rats (Fig. 4E).

Fig. 4. Concentration-response curves for ACh-induced responses of isolated rings of mesenteric arteries obtained from metformin-treated and untreated LETO and OLETF rats. Data were obtained in the absence (A) or presence (B–D) of the following drugs: 10 μM indomethacin + 100 μM L-NNA (B), 10 μM indomethacin + 100 nM apamin + 10 μM 1-[2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; C), or 100 μM L-NNA + 100 nM apamin + 10 μM TRAM-34 (D). E: concentration-response curves for the contractions induced by applying ACh in the presence of 100 μM L-NNA. Details are given in MATERIALS AND METHODS. Data are means ± SE. Number of determinations is shown in parentheses. *P < 0.05, **P < 0.01, ***P < 0.001 LETO group vs. OLETF group; *P < 0.05, **P < 0.01 untreated OLETF group vs. metformin-treated OLETF group; †P < 0.05, ††P < 0.01 untreated LETO group vs. metformin-treated LETO group.
dysfunction in diabetic arteries (5, 12, 30, 46), we examined the effects of chronic metformin treatment on endothelium-stimulated release of prostanoids in mesenteric arteries from LETO and OLETF rats (Fig. 5). ACh (10 μM) evoked releases of TxB2 (Fig. 5A), PGE2 (Fig. 5B), PGF2α, (Fig. 5C), and 6-keto-PGF1α (stable metabolite of prostacyclin; (D)) evoked by 10 μM ACh in mesenteric artery rings isolated from metformin-treated and untreated LETO and OLETF rats. Details are given in MATERIALS AND METHODS. Data are means ± SE. Number of determinations is shown in parentheses. *p < 0.05, **p < 0.01 vs. untreated LETO group; *p < 0.05, ***p < 0.001 vs. untreated OLETF group.

**Fig. 5. Release of prostanoids [TxB2 (stable metabolite of TxA2; A), PGE2 (B), PGF2α (C), and 6-keto-PGF1α (stable metabolite of prostacyclin; D)] evoked by 10 μM ACh in mesenteric artery rings isolated from metformin-treated and untreated LETO and OLETF rats.**

in the ACh-induced relaxation and contraction responses seen in mesenteric arteries from OLETF rats and 2) the improvements in those responses brought about by metformin treatment. First, we examined whether the expressions of COX, eNOS, and P-eNOS (Ser1177) proteins in mesenteric arteries might differ among the various groups. Immunoblot analysis of mesenteric arteries from all four groups (using anti-COX-1, -COX-2, -eNOS, and -P-eNOS antibodies) allowed detection of immunoreactive proteins (Fig. 6). The protein expression for COX-1 (Fig. 6A) was significantly greater in the OLETF group than in the LETO group (P < 0.05). The COX-2 level tended to be higher in OLETF rats than in LETO rats (although statistical significance was not reached) (Fig. 6B). The densitometric ratio of eNOS to β-actin was not different among the four groups of rats (data not shown), but that of P-eNOS to eNOS was significantly smaller in OLETF rats than in LETO rats (Fig. 6C). Unexpectedly, in OLETF rats, treatment with metformin did not significantly alter the expression levels of the COXs or P-eNOS (metformin-treated OLETF vs. untreated OLETF) (Fig. 6). However, the COX-2 level tended to be even higher in metformin-treated OLETF rats than in untreated OLETF rats (although statistical significance was not reached) (Fig. 6B).

**Evaluation of antioxidants and NO synthesis (as plasma nitrite and nitrate).** It is known that antioxidants play an important role in the prevention of cardiovascular diseases (3). The plasma level of NO3− (an oxidation product of NO) correlates with the level of NO biosynthesis (24), and the NO3−/NO2− ratio is often used as an indirect marker of oxidative stress (44). To investigate whether these plasma parameters were altered by chronic metformin treatment, we measured both the antioxidant capacity and the levels of NO3− and NO2− (as a measure of NO synthesis) in rat plasma. The plasma antioxidant level was lower in OLETF rats than in LETO rats (P < 0.01) (Table 2), and it was significantly higher in the metformin-treated OLETF group than in the untreated OLETF group (P < 0.01). The plasma NO3− level was lower in OLETF rats than in LETO rats, and the NO3−/NO2− ratio was significantly greater in OLETF rats than in LETO rats. The plasma NO3− level was similar between the two groups. In OLETF rats, metformin treatment had no significant effect on either the plasma levels of NO3− and NO2− levels or on the NO3−/NO2− ratio. However, both the plasma NO3− level and the NO3−/NO2− ratio were significantly higher in the metformin-treated OLETF group than in the untreated LETO group. No significant changes in antioxidant level, NO3− and NO2− levels, or NO3−/NO2− ratio were detected in nondiabetic (LETO) animals when they were treated with metformin.

**Quantification of superoxide anion by measurement of the amount of NBT reduced.** Since superoxide generation leads to enhancements of endothelium-dependent contractions (12, 46, 54, 60, 62) and metformin weakens oxidative stress (14, 28, 45), we examined the chronic effect of metformin on vascular superoxide production by measuring the amount of NBT reduced by superoxide (35, 37, 38) (Fig. 7). Superoxide production by the mesenteric artery was significantly greater in OLETF rats than in LETO rats. Chronic administration of metformin to OLETF rats significantly reduced this superoxide generation, but it had no effect in LETO rats.
DISCUSSION

Metformin is one of the most commonly used therapeutic drugs for patients with T2DM. It has been shown not only that metformin reduces hyperglycemia and improves insulin sensitivity but also that it has vasculoprotective effects (17, 20, 23, 29) that are largely independent of its well-known antihyperglycemic action. Despite the large number of studies published, it remains unclear which mechanisms underlie its vasculoprotective actions. In the present study, we examined whether the impaired endothelium-dependent vasomotor activity seen in mesenteric arteries isolated from established T2DM rats might be improved by acute and/or chronic treatment with metformin. To address the possible association between changes in endothelium-dependent relaxation and endothelium-dependent contraction in this artery, we chose OLETF rats as our chronic T2DM model. We did this because we and others have demonstrated that 1) OLETF rats manifest stable clinical and pathological features that resemble human T2DM (21), 2) abnormalities of vascular function are present in several vessels in OLETF rats (18, 33–35), and 3) there is recent evidence of an imbalance between endothelium-derived factors (viz. decreased EDRF signaling and increased EDCF signaling) in mesenteric arteries from OLETF rats (30).

Table 2. Plasma levels of antioxidants and NO metabolites (nitrite and nitrate) in metformin-treated and untreated LETO and OLETF rats

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>Metformin-Treated LETO</th>
<th>OLETF</th>
<th>Metformin-Treated OLETF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidants, mM</td>
<td>1.32±0.02 (7)</td>
<td>1.34±0.05 (7)</td>
<td>1.03±0.06 (7)†</td>
<td>1.28±0.05 (7)‡</td>
</tr>
<tr>
<td>Nitrite, nM</td>
<td>251.4±26.8 (9)</td>
<td>323.2±42.4 (7)</td>
<td>144.2±12.2 (9)†</td>
<td>165.8±31.2 (9)</td>
</tr>
<tr>
<td>Nitrate, μM</td>
<td>8.5±0.6 (9)</td>
<td>8.9±0.3 (7)</td>
<td>9.1±1.0 (9)</td>
<td>11.0±0.7 (9)*</td>
</tr>
<tr>
<td>Nitrate-to-nitrite ratio</td>
<td>35.7±3.1 (9)</td>
<td>29.8±2.9 (7)</td>
<td>66.4±9.1 (9)†</td>
<td>84.8±16.7 (9)*</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of determinations shown in parentheses. *P < 0.05, †P < 0.01 vs. LETO; ‡P < 0.01 vs. OLETF.
of eNOS increases NO production (27) and the plasma NO$_2$-to-NO$_3$ ratio was increased (vs. LETO). Chronic metformin treatment of OLETF rats did not alter these parameters, yet in such OLETF mesenteric arteries the ACH-induced NO-mediated relaxation was significantly reduced (vs. LETO) and metformin slightly (but significantly) improved this relaxation. Second, the OLETF mesenteric arteries exhibited abnormalities of endothelium-derived factor-mediated responses, for example, 1) ACH-induced relaxation was impaired, and the relaxation became weaker at higher concentrations of ACH; 2) ACH-induced EDHF-type relaxation was impaired (vs. LETO); and 3) the ACH-induced response under conditions in which COX activity is preserved was a contractile response rather than a relaxation, and this contraction was greater in OLETF rats than in LETO rats; and 4) both ACH-induced EDCF-mediated contraction and ACH-induced production of vasoconstrictor prostanoids (i.e., TxA$_2$ and PGE$_2$) were increased, as were the expressions of COX-1 and COX-2 (nonsignificantly) (vs. LETO). Metformin treatment markedly corrected these abnormalities without significantly altering the expressions of these COXs. Judging from these results, the metformin treatment-induced improvements of ACH-induced endothelium-dependent vasomotor responses in our OLETF mesenteric arteries can be largely attributed to suppression of vasoconstrictor prostanoids. Indeed, indomethacin enhanced the ACH-induced relaxation and eliminated the tendency for it to reverse at higher concentrations in OLETF mesenteric arteries. Moreover, previous studies have demonstrated that endothelium-dependent relaxation is modulated by vasoconstrictor prostanoids in a number of pathophysiological states (12, 60).

In our OLETF rats, plasma insulin, triglyceride, leptin, and glucose and serum NEFA concentrations were increased (vs. LETO). When we chronically administered metformin for 4 wk to such established OLETF rats, the metformin did not improve the blood insulin, triglyceride, leptin, or NEFA levels, but it did significantly lower blood glucose (however, the glucose level was still significantly higher than in the control LETO rats). Thus, at the dose given here (for 4 wk), this drug had vasculoprotective actions, even though the animals retained a degree of hyperglycemia and showed no significant improvements in metabolic parameters. Indeed, the present study has demonstrated that the direct effects of metformin administration (acutely, in vitro) are similar to those seen after chronic treatment in vivo. This suggests that its in vivo effect on hyperglycemia may not play a dominant role in the mechanism underlying the observed vasculoprotection. This is in accordance with findings made in previous studies (23, 29).

Although the precise mechanisms underlying the improvement of endothelial function seen upon chronic metformin treatment remain elusive, alterations in calcium handling could be involved. In fact, previous reports have suggested that accumulation of calcium within endothelial cells plays an important role in the release of EDCF (12, 60). Tang et al. (54) recently demonstrated that endothelial cells from spontaneously hypertensive rats (SHR) are more prone (vs. normotensive controls) to calcium overload and reactive oxygen species (ROS) overload upon stimulation with ACH, and that the sequence of events occurring during endothelium-dependent contractions requires an initial accumulation of calcium, which then activates COX and produces ROS along with EDCF.
which in turn stimulates thromboxane receptors, resulting in EDCF-mediated contraction. It is possible that the observed metformin treatment-induced suppression of vasoconstrictor prostanoids in OLETF mesenteric arteries is due to changes in intracellular calcium in the endothelium. This speculation is supported by the finding that metformin normalizes abnormal calcium handling in hepatocytes and ventricular myocytes (51, 58). Another possibility as to the mechanism underlying the above beneficial effects of metformin may be a modulation of oxidative stress. We (30) and others (46, 54) have reported that in diabetic arteries the increased oxidative stress facilitates and/or causes endothelium-dependent contraction by increasing the production of vasoconstrictor prostanoids. Interestingly, metformin exerts antioxidant effects by decreasing ROS production by vascular endothelial cells (14, 28, 45). In the present study, plasma antioxidant capacity was restored and mesenteric artery superoxide production was reduced in OLETF rats after chronic metformin treatment. Taking the above evidence together, we speculate that metformin’s beneficial effect on endothelial dysfunction in mesenteric arteries from OLETF rats may be at least partly due to a suppression of oxidative stress.

Prostacyclin is generally described as an endothelium-derived vasodilator, which by stimulating its receptor (i.e., prostacyclin receptors or IP receptors) induces smooth muscle relaxation (64). However, in mature SHR and their control Wistar-Kyoto (WKY) rats, neither prostacyclin nor its stable analog iloprost is able to produce a relaxation (15, 50). In the present study, the ACh-induced COX-mediated response was vasoconstriction rather than vasodilation. Moreover, beraprost (a stable prostacyclin analog) induced vasoconstriction rather than vasodilation in the mesenteric artery (data not shown). These results are consistent with prostacyclin contributing to endothelium-dependent contractions because IP receptors are no longer functional in our model (as in the above hypertensive model) (15, 50, 60). Moreover, in the present study, metformin treatment significantly reduced PGE_2 production and tended to reduce 6-keto-PGF_1α production in OLETF rats, even though COX-2 protein expression tended to be higher in metformin-treated OLETF rats than in untreated OLETF rats. These results suggest that COX-2 activity might not correlate with the expression of its protein in the metformin-treated OLETF mesenteric artery. Indeed, in several previous reports total COX-2 activity in tissues was regulated by posttranslational modification (such as S-nitrosylation) rather than by changes in its expression level (4, 22, 65). Although the mechanism by which metformin might regulate the level of COX-2 expression and/or activity remains unclear, the beneficial effects of metformin seen in our OLETF rats were not due to an increase in prostacyclin.

Hypertension is one of the major risk factors for cardiovascular diseases, and it is closely related to T2DM and insulin resistance (52). Metformin has been reported to lower blood pressure in a number of diabetic models (20, 61). This effect of metformin has been variously reported to be mediated by α-adrenergic blockade or ganglionic blockade (43), by an inhibition of sympathetic tone (47), by a direct effect on vascular smooth muscle (48), or by an opening of voltage-dependent potassium channels (29). Moreover, since metformin can improve several diabetic parameters (including hyperglycemia, hyperlipidemia, and insulin resistance), any of which could influence hypertension (23), the mechanisms that could conceivably underlie the antihypertensive action of metformin are complex. In the present study, the systolic blood pressure of the OLETF rats was higher than that of the age-matched LETO rats, and metformin lowered this parameter in OLETF rats without affecting either hyperglycemia or hyperinsulinemia. Although the blood pressure-lowering mechanism remains unclear, an improvement of EDHF signaling and a reduction in vasoconstrictor prostanoids might be more relevant than changes in metabolic parameters (viz. glucose and insulin) in mature OLETF rats. Since the contribution made by EDHF-mediated responses appears to be significantly greater in small than in large arteries (11), such responses may be important for blood pressure homeostasis. In fact, impaired EDHF-mediated responses have been demonstrated in several hypertensive models (11). Moreover, an increase in the prostanoids derived from COXs can contribute to increases in vascular tone and blood pressure both under physiological conditions (6) and in such pathophysiological states as hypertension (12, 60) and type 2 diabetes (5). Indeed, the development of fructose-induced hypertension in rats can be prevented by chronic thromboxane synthase inhibition without affecting either insulin sensitivity or the fructose-induced metabolic impairments (13). Furthermore, alterations in the activity of the SK_{Ca} channel—which are of crucial importance in the initiation of the EDHF signal after agonist stimulation (11, 32)—modulate arterial tone and blood pressure (55), and thromboxane receptor stimulation is associated both with loss of SK_{Ca} channel activity and with reduced EDHF responses in the rat mesenteric artery (8). Judging from these pieces of evidence, the blood pressure reduction seen in metformin-treated OLETF rats may be attributable both to improved EDHF signaling and to decreases in the production or release of vasoconstrictor prostanoids. In diabetic patients, even a small elevation in resting blood pressure significantly increases the risk of death from cardiovascular diseases, as well as the risks of myocardial infarction, congestive heart failure, and stroke (2). Indeed, each 10-mmHg increase in systolic blood pressure produces a 15% increase in the rate of death related to diabetes, as well as increases in the incidence of myocardial infarction (11%), congestive heart failure (12%), and stroke (19%) (2). On this basis, even a modest reduction in blood pressure during treatment with metformin could contribute to a significant decrease in diabetes-associated morbidity and mortality.

We conclude that in T2DM OLETF rats endothelial dysfunction is present in the mesenteric arteries (as indicated by reduced EDRF signaling and increased EDCF signaling) and that metformin corrects the imbalance between these endothelium-derived factors by reducing oxidative stress and suppressing the synthesis or release of vasoconstrictor prostanoids. These findings not only support the beneficial effects of metformin previously demonstrated in large intervention studies in T2DM patients but also offer a credible explanation for the beneficial effects that this drug has on the vascular system in T2DM.

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