Troglitazone Reduces Activity of the Na\(^+\)/H\(^+\) Exchanger in Fructose-Fed Borderline Hypertensive Rats

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Activation of the Na\(^+\)/H\(^+\) exchanger (NHE) is known to be related to elevated blood pressure in hyperinsulinemia. We previously demonstrated that a fructose-enriched diet induced hyperinsulinemia and hypertriglyceridemia, elevated NHE activity, increased intracellular calcium concentrations ([Ca\(^{2+}\)]\(_i\)), and increased blood pressure in borderline hypertensive rats (BHR). This study examines whether pharmacologically reducing plasma triglyceride or insulin concentrations lowers blood pressure and reduces NHE activity in fructose-fed BHR. Eicosapentaenoic acid (EPA), bezafibrate (BEZ), and troglitazone (TRO) were administered to treat hypertriglyceridemia and/or hyperinsulinemia. Rats were fed a 60% fructose diet or a control diet for 4 weeks, followed by a diet with either vehicle, EPA, BEZ, or TRO for 4 weeks. Intracellular pH (pHi) was measured in platelets by fluorescent dye. Platelet NHE activity was evaluated by the recovery of pHi following addition of sodium propionate (Vmax). [Ca\(^{2+}\)]\(_i\) in platelets were measured fluorometrically. In fructose-fed rats, EPA prevented further increase in blood pressure, and reduced triglyceride concentration and [Ca\(^{2+}\)]\(_i\) without affecting Vmax or plasma insulin concentrations. BEZ reduced triglyceride concentrations without affecting blood pressure, Vmax, [Ca\(^{2+}\)]\(_i\), or insulin concentrations. TRO prevented an increase in blood pressure, and reduced Vmax, [Ca\(^{2+}\)]\(_i\), and insulin, but not triglycerides. Plasma insulin and Vmax were positively correlated. In conclusion, improvement of hyperinsulinemia can decrease NHE activity and blood pressure in fructose-fed BHR. (Hypertens Res 2003; 26: 111–116)

Key Words: troglitazone, Na\(^+\)/H\(^+\) exchanger, hyperinsulinemia, hypertriglyceridemia, borderline hypertensive rats

Introduction

Metabolic and physiologic abnormalities associated with insulin resistance, such as hypertension, obesity, dyslipidemia, and glucose intolerance, may affect the vasculature, although it is still unclear whether hyperinsulinemia directly causes arteriosclerosis (1, 2). The Na\(^+\)/H\(^+\) exchanger (NHE), an integral membrane protein found in all eukaryotic cells, regulates intracellular pH (pHi), cell volume, and intracellular signaling in response to a variety of stimuli (3), and interacts with several receptors (4, 5). Recent reports have demonstrated a structural role for NHE in regulating the cortical cytoskeleton (6). NHE activity also has been associated with the regulation of blood pressure in several models (3, 7, 8). We previously examined the activities of NHE in fructose-induced metabolic abnormalities (9–11) in borderline hypertensive rats (BHR), Wistar-Kyoto rats (WKY), and spontaneously hypertensive rats (SHR). BHR were originally developed as a model of environmentally induced hypertension (12). We previously demonstrated that BHR fed a fructose diet developed hyperinsulinemia and hypertriglyceridemia, and showed elevations in NHE activity and blood pressure relative to those fed a standard diet (13). In this previous study, although fructose induced an equivalent degree of hyperinsulinemia in BHR, WKY, and SHR, the elevation in NHE occurred only in BHR. These findings suggest that hyperinsulinemia or hy-
pertriglyceridemia may be pivotal in activating the NHE in fructose-diet BHR. Although insulin may affect NHE activity, it remains unknown whether this effect is direct or indirect. Moreover, it is also unclear whether or not hypertriglyceridemia affects NHE activity. Therefore, in this study we used drug treatment of the metabolic abnormalities to regulate NHE activity and blood pressure. We studied three different drugs that are known to moderate metabolic abnormalities: eicosapentaenoic acid (EPA), bezafibrate (BEZ), and troglitazone (TRO). EPA and BEZ are used to treat hypertriglyceridemia, while TRO is effective against both hyperinsulinemia and hypertriglyceridemia.

Methods

Animals and Materials

Experiments were performed in male BHR. All experiments were performed in accordance with a protocol approved by the Guidelines for Animal Experimentation at Hyogo College of Medicine. All animals were housed under controlled lighting and temperature conditions. At 8 weeks of age, animals were randomly divided into two groups. The F group was fed a 60% fructose diet (60% fructose, 20% protein, and 5% fat; Oriental Yeast, Chiba, Japan) for 4 weeks, while the C group was fed a standard control diet for 4 weeks. Fructose feeding is widely used to induce insulin resistance (9–11). All rats were weighed and then subjected to systolic blood pressure measurement by the tail-cuff method using a UR5000 sphygmomanometer (Ueda, Tokyo, Japan). At 12 weeks of age, each group continued the same diet and was divided into 4 subgroups that were treated for 4 weeks with either EPA (1 g/kg/day in the ethyl ester form; Mochida, Tokyo, Japan), BEZ (100 mg/kg/day; Kissei Pharmaceutical, Tokyo, Japan), TRO (100 mg/kg/day; Sankyo, Tokyo, Japan) or saline vehicle (an untreated control subgroup). At 16 weeks of age, blood from all eight subgroups obtained as a measure of NHE activity (15). Platelets were resuspended in 1 ml of HEPES buffer at 37 °C to measure the baseline pHi using a fluorescence spectrophotometer (CAF110; Nihon Bunko, Tokyo, Japan). The wavelengths for excitation and emission were 500 and 540 nm, respectively; calibration was performed using the nigericin/high K⁺ method described by Thomas et al. (16). Then platelets were warmed to 37 °C in a Ca²⁺-containing buffer and maintained at an extracellular pH of 7.4.

The NHE was activated by the addition of a sodium propionate solution (final concentration, 100 mmol/l). The rate of pH recovery after this intracellular acidification with sodium propionate was computed using GraphPAD-Prism 2.0 software (Graph Pad Software, San Diego, USA) and expressed as dpHi/s. The rate constant of pHi recovery was obtained by iterative fitting of the experimental data to an exponential curve. The rate of initial pHi recovery (Vmax) was calculated as a measure of NHE activity (15). Determination was followed by disruption of the cells with 50 mmol/l digitonin. The fluorescence signal was calibrated using pH measurements following the addition of small amounts of HCl. We confirmed that no significant change in buffering capacity (17) occurred as a result of adding sodium propionate in any subgroup (C-vehicle, 16.0 ± 4.5 mmol/l; C-EPA, 11.8 ± 1.9; C-BEZ, 14.2 ± 1.1; C-TRO, 14.7 ± 4.7; F-vehicle, 12.8 ± 2.8; F-EPA, 12.4 ± 1.9; F-BEZ, 12.9 ± 1.6; F-TRO, 14.9 ± 4.6).

Preparation of Platelets

Platelet pellets were obtained from the platelet-rich plasma by centrifugation at 480 g for 30 min at room temperature (25 °C) and resuspended in 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) buffer solution (pH 6.5; NaCl, 140 mmol/l; KCl, 5 mmol/l; KH₂PO₄, 5 mmol/l; MgSO₄, 1 mmol/l; HEPES, 10 mmol/l; glucose, 5 mmol/l; and aspirin, 0.1 mmol/l at 25 °C). The final platelet density in the suspension was 1 × 10⁹/ml.

Determination of pH and Na⁺/H⁺ Exchanger Activity

pHi was measured according to a previously described method with slight modification, by adding a 1 mmol/l solution of a pH-sensitive fluorescent dye, 2-[7-(diethylamino)propyl]carboxyfluorescein acetoxyethyl ester (BCECF-AM; Dojindo, Kumamoto, Japan) (13, 15). The platelet suspension was incubated in a final concentration of 20 µmol/l BCECF-AM for 30 min at 37 °C. After centrifugation at 240 g for 10 min to remove extraneous dye, platelets were resuspended in 1 ml of HEPES buffer at 37 °C to measure the baseline pHi using a fluorescence spectrophotometer (CAF110; Nihon Bunko, Tokyo, Japan). The wavelengths for excitation and emission were 500 and 540 nm, respectively; calibration was performed using the nigericin/high K⁺ method described by Thomas et al. (16). Then platelets were warmed to 37 °C in a Ca²⁺-containing buffer and maintained at an extracellular pH of 7.4.

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[Ca²⁺]i Measurement

[Ca²⁺]i was measured according to the method described by Oshima et al. (14). Platelets were incubated with 2 µmol/l fura2/acetoxymethylester (Dojindo) for 30 min and analyzed with a fluorescence spectrometer using alternating excitation at 340 and 380 nm of ultraviolet light while measuring emission at 500 nm.
EPA, eicosapentaenoic acid; BEZ, bezafibrate; TRO, troglitazone; BW, body weight; TC, total cholesterol; TG, triglyceride. Values are the mean ± SEM. *p < 0.01 vs. control-fed rats administered vehicle, by ANOVA with the Bonferroni test; "p < 0.05 vs. control-fed rats administered vehicle, by Mann-Whitney U test; "p < 0.01 vs. control-fed rats receiving each treatment, by Mann-Whitney U test; ""p < 0.001 vs. control-fed rats administered vehicle, by ANOVA with the Bonferroni test; ""\( p < 0.01 \) vs. fructose-fed rats administered vehicle, by ANOVA with the Bonferroni test.

Table 2. Effects of EPA, BEZ, and TRO on pHı, Vmax, [Ca^2+]i, and in Control- and Fructose-Diet Borderline Hypertensive Rats

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<tr>
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<th>Control</th>
<th>Fructose</th>
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<tr>
<td>n</td>
<td>15</td>
<td>15</td>
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<tr>
<td>pHi (units)</td>
<td>7.03 ± 0.02</td>
<td>7.10 ± 0.04</td>
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<tr>
<td>Vmax (( \times 10^{-3} ) dpHi/s)</td>
<td>10.3 ± 0.3</td>
<td>13.2 ± 0.3*</td>
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<tr>
<td>[Ca^2+]i (nmol/l)</td>
<td>110.8 ± 3.3</td>
<td>143.4 ± 3.0*</td>
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EPA, eicosapentaenoic acid; BEZ, bezafibrate; TRO, troglitazone; pHı, intracellular pH; [Ca^2+]i, intracellular calcium concentration. Values are the mean ± SEM. *p < 0.001 vs. control-fed rats administered vehicle, by Mann-Whitney U test; "" \( p < 0.05 \) vs. control-fed rats administered TRO, by Mann-Whitney U test; "' \( p < 0.01 \) vs. fructose-fed rats administered vehicle, by ANOVA with the Bonferroni test; ""\( p < 0.05 \) vs. fructose-fed rats administered vehicle, by ANOVA with the Bonferroni test.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical analysis of data was performed using an Apple Macintosh computer with StatView software (Abacus Concepts, Cary, USA). Analysis of variance (ANOVA) followed by the Bonferroni test, repeated ANOVA, and the Mann-Whitney U test were used as appropriate. Two-tailed values of \( p < 0.05 \) were considered to indicate statistical significance. Correlations between exchanger activity and other parameters were calculated using the formula for Pearson’s correlation coefficient.

Results

Effects of the Fructose Diet and Drugs on Blood Pressure and Plasma Concentrations of Glucose, Insulin, and Lipids

We conducted our study of fructose-induced hyperinsulinemia for a period of 8 weeks to exclude any effect of obesity on blood pressure regulation. Before loading fructose, both systolic blood pressure and body weight were similar between BHR designated to receive the fructose and those designated to receive the control diet. After 4 weeks of a fructose or a control diet (i.e., at 12 weeks of age), the systolic blood pressure in the fructose-diet rats was greater than that in the control-diet rats (\( p < 0.05 \) by repeated ANOVA; Fig. 1). Treatment with EPA over the next 4 weeks prevented any further increase in systolic pressure in both groups, while TRO prevented a further increase in systolic pressure only in fructose-diet rats (Fig. 1). On the other hand, BEZ did not reduce blood pressure in rats fed either diet. Body weight, plasma glucose and cholesterol concentrations were similar in each diet group at 16 weeks of age (Table 1). The fructose diet increased plasma insulin concentrations relative to the control diet in the vehicle groups. TRO administration reduced plasma insulin concentrations in the fructose-diet rats to a level equal to that in the control-diet rats (Table 1), but EPA and BEZ administration did not. The fructose diet increased plasma triglyceride concentrations relative to the control diet in the vehicle groups. EPA and BEZ reduced plasma triglyceride concentrations in the fructose-diet rats. TRO tended to reduce plasma triglyceride concentrations in the fructose-diet rats, but the difference failed to reach the level of statistical significance. Among these agents, BEZ was the most powerful suppressor of the rise in plasma triglyceride concentrations.
Effects of the Fructose Diet on pHi and Vmax

Figure 2 shows the representative schema of pHi recovery in platelets from the control-diet and the fructose-diet rats. Because there was no difference in the initial pHi (Table 2) or buffering power (described in the Methods section) between the two groups of rats, we determined Vmax as a parameter of NHE activity. In the control-diet rats, Vmax was similar in platelets from the vehicle, EPA, BEZ, and TRO subgroups (Table 2). Vmax in the fructose-diet rats in the vehicle subgroup was higher than that in control-diet rats in the vehicle subgroup, suggesting that fructose feeding elevated the NHE activity. TRO administration reduced Vmax in the fructose-diet rats compared with the vehicle, but EPA and BEZ did not. There was no significant difference in Vmax in the fructose-diet rats between the EPA and BEZ subgroups.

Effects of the Fructose Diet on [Ca²⁺]

In the control-diet rats, the [Ca²⁺] values were similar in platelets from rats in the vehicle, EPA, BEZ, and TRO subgroups. [Ca²⁺] in the fructose-diet rats in the vehicle subgroup was higher than that in the control-diet rats in the vehicle subgroups. In the fructose-diet group, EPA and TRO administration reduced [Ca²⁺] compared with vehicle administration, although BEZ did not (Table 2).

Relationship between Plasma Insulin Concentration and Vmax

Plasma insulin concentrations showed a positive correlation with Vmax when all rats were considered together (Fig. 3).
TRO, which are insulin-sensitizing agents, have been shown to increase in blood pressure. Thiazolidinediones including troglitazone reduces plasma insulin concentrations and prevents any activity. One could hypothesize that hyperinsulinemia affects as a set point (17) in the metabolic abnormalities. 

In the present study we demonstrated that TRO reduced plasma insulin concentrations, [Ca^{2+}]_{i}, and NHE activity, further investigations will be needed to elucidate the detailed mechanisms by which TRO regulates [Ca^{2+}]_{i} and NHE activity.

N-3 polyunsaturated fatty acids, including EPA, can reduce serum triglyceride concentrations and blood pressure in stress- and salt-induced hypertension in BHR (26), as well as in hypertensive humans (27, 28). EPA can modulate intracellular Ca^{2+} signaling and intracellular cation transport associated with hypertension (29). In this study, we found that EPA lowered triglyceride concentrations, prevented further increases in blood pressure, and reduced [Ca^{2+}]_{i}, even though plasma insulin concentrations and NHE activity were not reduced. Accordingly, EPA may prevent an increase in blood pressure at least in part by mechanisms unrelated to insulin.

BEZ administration produced the greatest reduction in plasma triglyceride concentrations among the three drugs tested, but did not significantly affect NHE activity or blood pressure in fructose-fed BHR. Si et al. (30) demonstrated that fructose induced an elevation in blood pressure in Sprague-Dawley (S-D) rats—although the elevated blood pressure level was lower than that in the present study using BHR—and that BEZ reduced blood pressure levels in 40 days. Matsui et al. (31) showed that high-fructose + lard feeding, but not high-fructose alone, elevated blood pressure, and that BEZ improved insulin sensitivity in high-fructose + lard-fed S-D rats. Although hyperlipidemia may affect the membrane transport system by altering membrane fluidity via changing membrane phospholipid or eicosanoid composition, our data suggest that lowering triglyceride levels cannot significantly affect NHE activity, at least in fructose-fed BHR. This discrepancy is likely to reflect differences in specific animal models and experimental conditions; the body weights, blood pressures, and triglyceride concentrations all differed from those in the present study.

With respect to limitations of the present study, we did not determine whether fructose feeding together with drug treatment for longer than 4 weeks modulates NHE activity. Moreover, we studied only platelets, which predominantly express the NHE-1 isoform. To determine the significance of NHE activity in hyperinsulinemia, further investigations will be needed to determine which isoforms of NHE play important roles in blood pressure regulation (32, 33) and how pH changes affect the set point (17) in the metabolic abnormalities.

In summary, TRO administration prevented further increases in blood pressure and reduced NHE activity and plasma insulin concentrations. EPA prevented an increase in blood pressure and reduced triglyceride concentrations at least in part by mechanisms unrelated to insulin. BEZ administration sharply reduced triglyceride concentrations, but did not affect blood pressure or NHE activity. These results...
indicate that hyperinsulinemia may be linked to the activation of NHE, and that reduction of plasma insulin concentrations may be useful in the treatment of hypertension.

References