Caffeine Intake Improves Fructose-Induced Hypertension and Insulin Resistance by Enhancing Central Insulin Signaling

Tung-Chen Yeh, Chun-Peng Liu, Wen-Han Cheng, Bo-Rong Chen, Pei-Jung Lu, Pei-Wen Cheng, Wen-Yu Ho, Gwo-Ching Sun, Jau-Cheng Liou, Ching-Jiunn Tseng

Abstract—Recent clinical studies found that fructose intake leads to insulin resistance and hypertension. Fructose consumption promotes protein fructosylation and formation of superoxide. In a previous study, we revealed that inhibition of superoxide production in the nucleus tractus solitarii (NTS) reduces blood pressure. Caffeine displays significant antioxidant ability in protecting membranes against oxidative damage and can lower the risk of insulin resistance. However, the mechanism through which caffeine improves fructose-induced insulin resistance is unclear. The aim of this study was to investigate whether caffeine consumption can abolish superoxide generation to enhance insulin signaling in the NTS, thereby reducing blood pressure in rats with fructose-induced hypertension. Treatment with caffeine for 4 weeks decreased blood pressure, serum fasting glucose, insulin, homeostatic model assessment-insulin resistance, and triglyceride levels and increased the serum direct high-density lipoprotein level in fructose-fed rats but not in control rats. Caffeine treatment resulted in the recovery of fructose-induced decrease in nitric oxide production in the NTS. Immunoblotting and immunofluorescence analyses further showed that caffeine reduced the fructose-induced phosphorylation of insulin receptor substrate 1 (IRS1^S307) and reversed Akt^S473 and neuronal nitric oxide synthase phosphorylation. Similarly, caffeine was able to improve insulin sensitivity and decrease insulin levels in the NTS evoked by fructose. Caffeine intake also reduced the production of superoxide and expression of receptor of advanced glycation end product in the NTS. These results suggest that caffeine may enhance insulin receptor substrate 1-phosphatidylinositol 3-kinase-Akt-neuronal nitric oxide synthase signaling to decrease blood pressure by abolishing superoxide production in the NTS. (Hypertension. 2014;63:00-00.) • Online Data Supplement

Key Words: caffeine • fructose • hypertension • nitric oxide • solitary nucleus

In humans, the intake of sugar-sweetened beverages is correlated with the prevalence of type 2 diabetes mellitus and obesity. High fructose intake is used as a well-established animal model for insulin resistance. Some studies have shown that chronic fructose intake in normal rats induces hypertension in association with insulin resistance. However, insulin infusion was found to stimulate local vasodilation by enhancing the action of nitric oxide (NO). The effect of insulin on the baroreceptor reflex in nucleus tractus solitarii (NTS) is probably mediated by a change in sympathetic nervous activity. Furthermore, sympathectomy can prevent the development of fructose-induced hypertension in rats. Recent studies suggest that fructose consumption increases superoxide generation and attenuates baroreflex response. Interestingly, inhibition of superoxide generation in NTS can reduce blood pressure (BP) in stroke-prone hypertensive rats. The NTS is located in the dorsal medulla of the brain stem, which is the primary integrating center for cardiovascular regulation and other autonomic functions of the central nervous system. Furthermore, NO has important modulatory functions in the NTS, including the modulation of arterial BP and sympathetic nerve activity. In previous experiments, we demonstrated that insulin microinjected into the NTS induces a depressor effect and initiates an interaction between insulin and phosphatidylinositol 3-kinase...
(PI3K)-Akt-neuronal NO synthase (nNOS)-NO–mediated signaling in the NTS.4

Caffeine is most likely the world’s most frequently ingested pharmacological substance. Epidemiological studies have shown that long-term caffeine consumption diminishes diabetic symptoms by enhancing insulin sensitivity through more efficient insulin signaling.11,12 However, although some studies have shown that acute caffeine consumption elevates BP, other studies have shown that habitual coffee intake is less likely to increase BP.13,14 In fact, current studies have suggested a protective role of coffee intake against hypertension.14,15 Nevertheless, the relationship between fructose-induced hypertension and caffeine-antagonized hypertension has not been established. Therefore, we tested the hypothesis that caffeine can inhibit fructose-induced hypertension.

In the present study, we examined whether superoxide could be inhibited by administering caffeine. In addition, we investigated the downstream signaling pathway involved in the effect of caffeine on the NTS. Our results suggest that a defect in the insulin receptor substrate 1 (IRS1)-PI3K-Akt-nNOS signaling pathway in the NTS is involved in the superoxide-mediated modulation of hypertension of fructose-fed rats. However, the most interesting finding of this study was that caffeine may reverse the defect in the insulin signaling pathway through reduction of superoxide generation.

Materials and Methods
Additional experimental detail is provided in the online-only Data Supplement.

Results
Consumption of Fructose Induces Metabolic Indicators in Rats
The Table provides the body weight and BP, fasting plasma glucose, insulin, triglyceride, high-density lipoprotein, plasma norepinephrine, and cholesterol levels measured in the experimental groups. As recently reported from a previous study,16 our results revealed significant elevation of serum triglyceride concentrations in the fructose group compared with the control group. Fasting blood glucose was higher in the fructose group. Furthermore, serum insulin and homeostatic model assessment-insulin resistance index were elevated in fructose-fed rats, whereas direct high-density lipoprotein level was significantly decreased. As shown in the Table, fructose administration significantly increased systolic BP and mean BP compared with the control group. There was no difference in body weight after fructose treatment compared with the control group.

Caffeine Prevents Fructose-Mediated Metabolic Defects
The levels of fasting glucose, insulin, homeostatic model assessment-insulin resistance, norepinephrine, and triglycerides were significantly reduced, whereas direct high-density lipoprotein levels were increased in the fructose-cafﬁne group compared with the fructose group. These results indicate that caffeine can suppress fructose-induced hypertension.

Caffeine Inhibits Fructose-Induced Impairment of NO Production in the NTS
Interestingly, NO production in the NTS was found to decrease significantly after fructose administration (Figure 1A, lanes 1 and 2). Caffeine stimulates increased central NO production in the brain.17 Our findings revealed that treatment with caffeine resulted in the recovery of fructose-induced decrease of NO production in the NTS (Figure 1A, lanes 2 and 3). Immunoblotting analysis demonstrated that fructose could significantly decrease nNOS phosphorylation in the NTS (Figure 1B and 1C, lanes 1 and 2). Furthermore, immunofluorescence staining against phosphorylated nNOS[S1416] in the NTS revealed that fructose reduced the number of phosphorylated nNOS[S1416]-positive cells compared with the control group (Figure 1D and 1E, lanes 1 and 2). We performed immunoblotting and immunofluorescence analyses to determine whether caffeine pretreatment could restore nNOS[S1416] phosphorylation in the NTS of fructose-fed rats (Figure 1B–1E, lanes 2 and 3). However, immunofluorescence and immunoblotting analyses showed no significant difference in nNOS[S1416].

Table. General Characteristics of Participants in the 4 Groups of Rats

<table>
<thead>
<tr>
<th>Parameter/Group</th>
<th>Control (n=6)</th>
<th>Fructose (n=6)</th>
<th>Fructose+Caffeine (n=6)</th>
<th>Caffeine (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>245±6.8</td>
<td>244.8±4.6</td>
<td>243.5±5.5</td>
<td>240.3±4.9</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>126.6±2.8</td>
<td>155.7±4.9*</td>
<td>133.7±1.6†</td>
<td>129.7±2.4</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>112.5±0.6</td>
<td>134.2±3.9*</td>
<td>113.6±0.1†</td>
<td>109.6±2.8</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>70.8±7.4</td>
<td>331.5±39.7*</td>
<td>208.3±15†</td>
<td>108.2±15.2</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>5.43±0.79</td>
<td>176.1±25*</td>
<td>85.4±14.7†</td>
<td>5.83±0.95</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.13±0.013</td>
<td>14.73±0.9*</td>
<td>10.8±1.8†</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>dHDL, mg/dL</td>
<td>72.0±0.4</td>
<td>61.0±2.5*</td>
<td>70.9±2.4†</td>
<td>72.2±2.0</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>59.6±2.4</td>
<td>124.4±8.7*</td>
<td>71.5±7.1†</td>
<td>62.0±4.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>117.2±4.3</td>
<td>104.9±4.2</td>
<td>117.4±4.4</td>
<td>120.7±2.1</td>
</tr>
<tr>
<td>Plasma norepinephrine, pg/mL</td>
<td>333.3±209</td>
<td>1139.8±78.2*</td>
<td>406.8±247.5†</td>
<td>243.3±59.6</td>
</tr>
</tbody>
</table>

Body weights, levels of serum triglycerides, serum norepinephrine, fasting serum glucose, and fasting serum insulin, systolic blood pressure, mean blood pressure, and dHDL cholesterol levels were determined in fructose- or caffeine-treated rats. The presented values are mean±SEM. dHDL indicates direct high-density lipoprotein; and HOMA-IR, homeostatic model assessment-insulin resistance.

*P<0.05 vs control group.
†P<0.05 vs fructose group.
phosphorylation between the caffeine group and the control group (Figure 1B–1E, lanes 1 and 4).

Caffeine Attenuates Fructose-Induced Insulin Signaling Defects in the NTS

Our previous studies demonstrated that nNOS is a downstream factor of insulin-PI3K-Akt signaling–mediated central BP regulation in the NTS. 18 We further tested whether caffeine might activate the Akt-nNOS cascade in the NTS. Immunoblotting analysis revealed significantly decreased AktS473 phosphorylation in the NTS of fructose-fed rats compared with controls (Figure 2A and 2B, lanes 1 and 2). After the administration of caffeine, a significant increase in AktS473 phosphorylation was observed in the fructose-fed rats (Figure 2A and 2B, lanes 2 and 3), whereas the level in the caffeine group was unchanged compared with the control group (Figure 2A and 2B, lanes 1 and 4).

Previously, we provided evidence that microinjection of insulin in the NTS induces depressor effects in normotensive Wistar-Kyoto rats. 4 To confirm whether caffeine influences the relationship between insulin and BP regulation in the NTS, we microinjected insulin into the NTS and monitored the resultant changes in BP. Microinjection of insulin into the NTS produced prominent depressor effects in controls (Figure 2C and 2D, lane 1). Similarly, significant depressor effects were observed in caffeine group rats after insulin injection (Figure 2C and 2D, lane 4). The depressor response to insulin was significantly attenuated by fructose administration (Figure 2C and 2D, lane 2). However, the insulin-induced depressor effect in the NTS was restored after caffeine treatment in the fructose group (Figure 2C and 2D, lane 3).

We then investigated whether the administration of fructose and caffeine influences the insulin content in the NTS. Figure 3E shows that a significant decrease in the insulin level was observed in comparison with fructose (Figure 2E, lanes 1 and 2). After treatment with caffeine for 4 weeks, the insulin concentration was reduced significantly in the fructose group (Figure 2E, lanes 2 and 3). However, the analysis of insulin level showed no significant differences in the NTS between the control and caffeine groups (Figure 2E, lanes 1 and 4).

A recent study indicated that fructose-induced insulin resistance increases the phosphorylation of IRS1 at serine 307. 19 Therefore, we further investigated whether IRS1S307 phosphorylation participates in the insulin signaling defect induced by fructose in the NTS. The results of the immunoblotting analysis demonstrated that fructose increased IRS1S307 phosphorylation in the NTS compared with the controls (Figure 2F and 2G, lanes 1 and 2). As expected, we observed that caffeine inhibited IRS1S307 phosphorylation in the NTS of the fructose group (Figure 2F and 2G, lanes 2 and 3). However, the phosphorylation of IRS1S307 was not significantly different between the caffeine and control groups (Figure 2F and 2G, lanes 1 and 4).

Caffeine Inhibits Fructose-Induced Superoxide Generation and Receptor of Advanced Glycation End-Product Expression in the NTS

Previous research has shown that superoxide induces phosphorylation at serine 307 of IRS1. 20 Therefore, we investigated the in situ level of superoxide in the NTS to establish a potential association between fructose and superoxide production. In the present study, the cells in the NTS of the fructose group rats exhibited significantly higher dihydroethidium fluorescence activity compared with control group rats (Figure 3A

Figure 1. Caffeine ameliorates the fructose-induced impairment of nitric oxide (NO) production and increased neuronal NO synthase (nNOS) phosphorylation in the nucleus tractus solitarii (NTS). A, Administration of fructose results in low levels of NO. Systemic treatment with caffeine leads to significantly elevate NO levels in the NTS in the fructose group compared with the fructose controls. B, Immunoblots depicting low levels of phosphorylated (P)-nNOSS1416 protein in the NTS of the fructose group compared with the control group (lanes 1 and 2). Caffeine treatment increases the level of P-nNOSS1416 protein in the fructose group (lanes 3). C, The results of densitometric analyses of P-nNOSS1416 levels are shown before and after treatment with fructose or caffeine. D, Representative green fluorescence images of P-nNOSS1416 cells in the NTS before and after systemic administration of fructose or caffeine. The nuclei of the cells in the NTS were counterstained with 4',6-diamidino-2-phenylindole and exhibit blue fluorescence. Note the reduced intensity of green fluorescence observed in the NTS sections in the fructose group compared with other groups. E, The bar graphs are representative of the P-nNOSS1416 fluorescence intensity in the NTS of the indicated groups. Note the significantly greater P-nNOSS1416 fluorescence intensity in the NTS of the fructose-caffeine group. The presented values are mean±SEM (n=6). *P<0.05 vs the control group; #P<0.05 vs the fructose group.
intake significantly increased RAGE expression in the NTS (Figure 3C and 3D, lanes 2). Conversely, after caffeine treatment, the fructose-fed rats displayed a significant decrease in RAGE expression compared with the fructose rats (Figure 3C and 3D, lanes 2 and 3). No change in RAGE expression was observed in the caffeine group rats compared with the control group rats (Figure 3C and 3D, lanes 1 and 4).

Discussion

Recently, epidemiological reports have suggested that dietary fructose intake promotes hyperlipidemia, hyperinsulinemia, and hypertension.21,22 Our findings extend these observations to fructose-fed rats, which show similar metabolic disturbances.14,23 In the present study, fructose intake caused increased levels of serum norepinephrine, serum triglycerides, insulin, and fasting serum glucose and reduced high-density lipoprotein but resulted in no change in body weight or cholesterol compared with control rats. However, there is some controversy about fructose-induced hypertension, mainly related to the potential to develop hypertension, and many potential explanations for the discrepancy in these findings have been offered, such as differences in the techniques used to measure BP and the feed or drinking conditions.24 In particular, most studies have found that administering rats 10% fructose in drinking water induces a more severe metabolic disorder compared with providing 60% fructose in food.22,24 In this study, we used 10% fructose in drinking water as a fructose-induced hypertension model. Our data revealed the presence of hypertension in fructose-fed rats.

Previous studies have demonstrated that the development of hypertension is associated with insulin resistance and hyperinsulinemia.21,25 Using fructose-fed rats as an animal model of insulin resistance, several studies have revealed increased superoxide generation.7,21 Furthermore, hyperinsulinemia could activate the sympathetic nervous system, which in turn will elevate the BP.20 These observations demonstrate that the existence of a functional sympathetic nervous system is required for the development of elevated BP and plasma insulin levels in fructose-fed rats.27 Furthermore, in a clinical setting, derangements of the peripheral endothelial NO system have been related to the development of hypertension.28 It may be a pathogenic factor for abnormal regulation of vascular tones through imbalance of NO and superoxide production, which may be associated with the development of hypertension in the insulin-resistant state.29 However, Liu et al23 demonstrated that the vascular NO pathway may not be causally related to the development of fructose-induced hypertension.28 In a previous study, we showed that angiotensin II induces superoxide generation, which impairs NO production in the NTS.30 The depressor effect of NO in the NTS occurs through the inhibition of sympathetic nervous activity.30 Our findings extend previous observations that central insulin resistance in the NTS might participate in a superoxide-dependent pathway via the impairment of NO production to produce hypertension of fructose-fed rats.

High-fructose diets increase the phosphorylation of serine 307 on IRS1 in rats.19 Increased serine phosphorylation on IRS1 decreases the association of IRS1 with the insulin receptor and inhibits PI3K activity, thereby inhibiting insulin

and 3B, lanes 1 and 2). However, caffeine treatment reduced fructose-induced dihydroethidium fluorescence activity in the NTS (Figure 3A and 3B, lanes 2 and 3), whereas no significant difference was observed between caffeine and controls (Figure 3A and 3B, lanes 1 and 4). We then investigated whether fructose influenced receptor of advanced glycation end product (RAGE) expression in the NTS. The results of the immunofluorescence analysis showed that fructose

Figure 2. Fructose induces insulin signaling pathway defect in the nucleus tractus solitarii (NTS). A, Immunoblot depicting low levels of phosphorylated (P)-AktS473 protein in the NTS of the fructose group compared with the control group (lanes 1 and 2). Caffeine treatment increases the level of P-AktS473 protein in the fructose group (lane 3). B, Densitometric analysis of P-AktS473 protein levels before and after treatment with fructose or caffeine. C, Representative tracings demonstrating the cardiovascular effects of insulin in the unilateral NTS of anesthetized rats. The depressor effects of insulin are significantly attenuated by fructose administration. After caffeine treatment in the fructose group, the depressor effects of insulin are recovered. D, Summarized data are shown in the graph, revealing the effects on mean blood pressure (MBP) after the microinjection of insulin into unilateral NTS. E, Fructose treatment results in high levels of insulin, whereas systemic treatment with caffeine leads to significantly decreased insulin levels in the NTS in the fructose group compared with fructose controls. F, Immunoblots depicting low levels of P-insulin receptor substrate 1 (IRS1)S307 protein in the NTS in the fructose group compared with the control group (lanes 1 and 2). Caffeine treatment increases the level of P-IRS1S307 protein in the fructose group (lane 3). G, Densitometric analysis of P-IRS1S307 protein levels before and after treatment with fructose or caffeine. The values indicated with bars are mean±SEM (n=6). *P<0.05 vs control and #P<0.05 vs fructose.
Nicotinamide adenine dinucleotide phosphate oxidase activation may participate in the regulation of central insulin resistance by fructose consumption. The results of the present study suggested that caffeine treatment does not induce hypertension. Interestingly, caffeine treatment was able to abolish fructose-induced hypertension. Furthermore, our data showed that the A2a-extracellular signal–regulated protein kinase 1/2–endothelial NOS pathway may not participate in the regulation of central BP by caffeine or fructose in rats. Thus, we speculate that caffeine intake may abolish the hypertension induced by fructose through other mechanisms. However, further studies are needed to verify this signaling mechanism in the NTS.

Several studies have suggested regulatory roles of caffeine associated with its biological effects. First, caffeine has been demonstrated to stimulate increased central NO production in the brains of mice. Second, because caffeine is an antioxidant, regular caffeine intake prevents diet-induced insulin resistance. Third, caffeine can activate Akt phosphorylation. Fourth, caffeine intake did not affect circulating insulin secretion significantly. Finally, caffeine does not alter the phosphorylation of the insulin receptor, IRS1-associated PI3K, or Akt. These findings suggest the possibility of a broad physiological role for caffeine. In the present study, we observed that caffeine could improve insulin sensitivity and decrease insulin level in the NTS of fructose-fed rats. However, the mechanism through which caffeine enhances insulin sensitivity is not clear. Our current findings provide further support for these observations by showing that caffeine may upregulate IRS1-PI3K-Akt-nNOS signaling.

In addition, our previous findings showed that insulin-PI3K-Akt-nNOS signaling in the NTS is linked to NO production and BP regulation. The present study provides further support for these observations by showing that the IRS1-PI3K-Akt-nNOS signaling defect in the NTS is involved in fructose-associated hypertension. Furthermore, the dietary fructose–mediated generation of advanced glycation end products activation and expression of RAGE contribute to metabolic syndrome. However, activation of nicotinamide adenine dinucleotide phosphate oxidase by advanced glycation end product–mediated superoxide generation led to altered gene expression via RAGE. Nicotinamide adenine dinucleotide phosphate oxidase was a major source of superoxide generation because its activation was associated with impaired bioavailability of NO. The findings of this study are further supported by reports that RAGE-mediated nicotinamide adenine dinucleotide phosphate oxidase activation may participate in the regulation of central insulin resistance by fructose in rats. Nevertheless, in the present study, we also observed that caffeine may inhibit fructose-induced superoxide generation caused by RAGE-mediated nicotinamide adenine dinucleotide phosphate oxidase activation.

Fructose consumption is known to influence adenine nucleotide catabolism and ATP at the cellular level. However, caffeine is a known competitive antagonist of adenosine receptors. Our previous findings demonstrated that the adenosine A2a-extracellular signal–regulated protein kinase 1/2–endothelial NOS signaling pathway in the NTS mediates NO production associated with the regulation of BP. Interestingly, long-term caffeine consumption diminishes diabetic symptoms by enhancing insulin sensitivity through more efficient insulin signaling. In the present study, we also observed that caffeine may alleviate fructose-induced metabolic disturbances. However, some researchers have concluded that caffeine consumption may elevate BP, leading to a higher risk of hypertension. Conversely, most epidemiological studies have shown that the habitual intake of caffeinated coffee does not increase the risk of hypertension, and chronic caffeine intake has been found to prevent diet-induced hypertension. Corti et al also demonstrated that in habitual coffee drinkers, there is no significant change in systolic BP after coffee consumption. The results of the present study suggested that caffeine treatment does not induce hypertension. Interestingly, caffeine treatment was able to abolish fructose-induced hypertension.

Figure 3. Caffeine prevents superoxide and receptor of advanced glycation end product (RAGE) overexpression in the nucleus tractus solitarii (NTS) after fructose stimulation. A, Representative red fluorescence images of superoxide-producing cells in the NTS after systemic administration of fructose or caffeine. Note the reduced numbers of red fluorescent cells in the NTS sections from the caffeine-treated groups compared with the fructose group. B, The superoxide index is the relative mean fluorescence intensity of dihydroethidium. Note the significant decrease in superoxide production in the NTS in the fructose-caffeine group. C, Representative green fluorescence images of RAGE cells in the NTS before and after systemic administration of fructose or caffeine. The nuclei of the cells in the NTS were counterstained with 4',6-diamidino-2-phenylindole and exhibit blue fluorescence. Note the reduced intensity of green fluorescence in the NTS sections from the fructose group compared with other groups. D, Bar graphs indicate the RAGE fluorescence intensity in the NTS in the indicated groups. Note the significantly higher RAGE fluorescence intensity in the NTS in the fructose-caffeine group. The presented values are mean±SEM (n=6). *P<0.05 vs the control group; #P<0.05 vs the fructose group.
signaling. We also observed that caffeine stimulates NO production in the NTS to reduce fructose-induced hypertension. Taken together, these results suggest that caffeine may reverse the defect in the insulin signaling pathway to increase NO production in the NTS. However, the mechanism connecting caffeine and insulin signaling in the NTS remains unknown. Further studies are needed to verify the downstream pathways that mediate the effect of caffeine in enhancing insulin sensitivity and the prevention of hypertension.

In conclusion, we have suggested in this report that caffeine may enhance insulin sensitivity in the NTS to prevent fructose-induced hypertension by increasing NO production. Furthermore, we showed that caffeine may upregulate IRS1-PI3K-Akt-nNOS signaling via reduced superoxide generation to enhance NO production in the NTS. This is the first study to demonstrate the mechanism through which caffeine may increase insulin sensitivity in the NTS and reduce high BP.

Perspectives
Both insulin resistance and superoxide generation have been identified as underlying pathogenic mechanisms associated with hypertension. In this study, we demonstrated the importance of fructose and caffeine in the NTS in mediating superoxide-associated central insulin resistance. We also investigated the cross talk between caffeine and fructose in the pathogenesis of hypertension. Whether this interplay between the systemic administration of fructose and caffeine takes place in other areas of the brain involved in the maintenance of central cardiovascular regulation also warrants investigation. Much more work will be necessary to understand the role of caffeine in hypertension for potential applications in therapeutic strategies.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is New?**

- This is the first study showing that the insulin receptor substrate 1-phosphatidylinositol 3-kinase-Akt-neuronal nitric oxide synthase insulin signaling defect is involved in superoxide-induced hypertension in the nucleus tractus solitarii stimulated by fructose intake. However, the most interesting result of this work is that caffeine may reverse the fructose-induced defect in the insulin signaling pathway by reducing superoxide generation.

**What Is Relevant?**

- Reactive oxygen species participate in the central autonomic networks involved in fructose-induced insulin resistance. Caffeine has been reported to increase central nitric oxide production in the brain. Furthermore, caffeine ameliorates diabetic symptoms by enhancing insulin sensitivity.

**Summary**

This study provides the first direct evidence that caffeine enhances insulin receptor substrate 1-phosphatidylinositol 3-kinase-Akt-neuronal nitric oxide synthase signaling in the nucleus tractus solitarii to prevent fructose-induced hypertension, possibly via decreasing superoxide production. Our findings provide new insights into the central nervous system regulation of hypertension and may contribute to the development of therapies against this disease.
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Caffeine intake improves fructose-induced hypertension and insulin resistance by enhancing central insulin signaling

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Running Title: Caffeine attenuate fructose-induced hypertension
MATERIALS AND METHODS

Experimental chemicals
All experimental drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA) except when otherwise noted.

Animals
Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees. Animal experiments were performed on normotensive male Wistar-Kyoto (WKY) rats at 6 weeks of age, which were obtained from the National Science Council Animal Facility (Taipei, Taiwan). They were housed in an animal room in the Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). The rats were randomly divided into the following 4 groups: (1) a control group of WKY rats; (2) a fructose group consisting of WKY rats fed 10% fructose in drinking water; (3) a fructose-caffeine group composed of WKY rats fed 10% fructose in drinking water with caffeine; and (4) a caffeine group composed of WKY rats fed drinking water with caffeine. The fructose group was administered 10% fructose in their drinking water for 4 weeks. The fructose-caffeine group was given caffeine (15 mg/kg/day, which corresponds to approximately 3-4 cups of coffee/day) via gavage for 4 weeks. Different doses of caffeine were administered via gavage. All subsequent doses (1.5 mg, 5 mg and 15 mg) suppressed the hypertension induced by fructose. The administration of caffeine (15 mg) significantly suppressed the pressor effect induced by fructose (data not shown). All rats were provided with normal rat chow (Purina, St. Louis, MO, USA) and tap water ad libitum. Proper and sufficient food, water, shelter, exercise, veterinary care, and humane treatment were provided at all times. During each measurement, 10 individual readings were obtained in a rapid sequence. The highest and lowest readings were excluded, and the remaining 8 readings were averaged. Additional experimental detail is provided in the online-only Data Supplement.

Radio-Telemetry Monitoring of Blood Pressure
The Dataquest IV telemetry system (Data Sciences International, New Brighton, Minnesota) was used for the measurement of systolic pressure, diastolic pressure, mean arterial pressure, and heart rate, as previously described. The monitoring system consists of a transmitter (a radio frequency transducer model TA11PA), receiver panel, consolidation matrix, and personal computer with accompanying software. Before the device was implanted, calibrations were verified for accuracy within 63 mmHg. Rats at 8 weeks of age were anesthetized with Zoletil 50. The flexible catheter of the transmitter was surgically secured in the abdominal aorta just below the renal arteries and pointed upstream (against the flow), and the transmitter was sutured to the abdominal wall. The rats were housed in...
individual cages following the operation. For data acquisition, each cage was placed over a
receiver panel, which was connected to the personal computer.

The experimental rats were unrestrained and free to move within their cages. Hemodynamic data were sampled every 5 minutes for 10 seconds. Preliminary experiments showed that the blood pressure and heart rate of the rats stabilized 7 days after the operation. Therefore, each drug treatment was commenced 7 days after surgery, and telemetry data were collected for 28 days of treatment.

Immunoblotting Analysis

The NTS was dissected via micropunch (1 mm inner diameter) from a 1 mm-thick brainstem slice at the level of the obex under a microscope. Briefly, total protein was prepared by homogenizing the NTS for 1 hour at 4°C in lysis buffer, a protease inhibitor cocktail, and phosphatase inhibitor cocktail 2 (all purchased from Sigma-Aldrich). Protein extracts (20 µg/sample assessed through a BCA protein assay, Pierce Chemical Co., IL, USA) were subjected to 6-12.5% SDS-Tris glycine gel electrophoresis and transferred to a polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, UK). The membranes were blocked and incubated at 4°C overnight with the appropriate antibody: anti-P-IRS1, anti-IRS1 (Millipore, Billerica, MA, USA); anti-P-Akt, anti-Akt (Cell Signaling Technology, Danvers, MA, USA); anti-P-nNOS, anti-nNOS (Abcam, Cambridge, UK); or anti-RAGE (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

In Situ Detection of Superoxide in the NTS

Endogenous in vivo superoxide production in the NTS was determined via dihydroethidium (DHE; Invitrogen, Carlsbad, CA, USA) staining. The NTS was dissected, quickly frozen, embedded in OCT, and then placed in liquid nitrogen. Cryostat slices (30 µm) were stained in the dark for 30 min at 37°C with 1 µM DHE. The samples were analyzed using confocal microscopy (Carl Zeiss LSM 5 PASCAL, Göttingen, Germany).

Measurement of NO in the NTS

Samples were deproteinized with Microcon YM-30 centrifugal filter units (Millipore). The amount of total NO in the samples was determined using a modified protocol based on the purge system of the Sievers Nitric Oxide Analyzer (NOA 280i; Sievers Instruments, Boulder, CO, USA) involving chemiluminescence. The samples (5 µl) were injected into a reflux column containing 0.1 mol/L VCl₃ in 1 mol/L HCl at 90°C to reduce any nitrates or nitrites (NOx) to NO. NO was then combined with the O₃ produced by the analyzer to form NO₂. The resulting emissions from the excited NO₂ were detected by a photomultiplier tube and recorded digitally (mV). The values were then plotted on a standard curve of NaNO₃.
concentrations determined simultaneously. Measurements were collected in triplicate for each sample.

**Measurement of Insulin in the NTS**

NTS insulin levels were measured using the Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden) and detected using the Biochrom Anthos Zenyth 200rt Microplate Reader (Cambridge, UK).

**Intra-NTS Microinjection**

The preparation of animals for intra-NTS microinjection and the methods used to locate the NTS have been described previously. Briefly, a polyethylene cannula was inserted into the femoral vein for fluid supplementation. Blood pressure (BP) was measured via a femoral artery cannula with a pressure transducer and polygraph (Gould, Cleveland, OH, USA). To verify that the needle tip of the glass electrode was positioned accurately in the NTS, groups of animals were first injected with insulin (3.5 mg/mL, Novo Nordisk, Bagsvaerd, Denmark) in the unilateral NTS, and then, L-glutamate (0.154 nmol/60 nL) was microinjected.

**Immunofluorescence Staining Analysis**

The rats were perfused first with saline, then with 4% formaldehyde, and finally with 30% sucrose. Brain stem sections (20 µm thick) were stained with cresyl violet, and proper placement of the pipette tip in the NTS was verified by examining the sections under a microscope. The brain stem sections were incubated with rabbit anti-phospho-nNOS (1:100; Millipore) and rabbit anti-RAGE (1:50; Santa Cruz Biotechnology, Santa Cruz) antibodies. After washing the sections with PBS, they were incubated with an Alexa Fluor 488 donkey anti-rabbit IgG (1:200; Invitrogen) antibody at 37°C for 2 hours. The sections were finally analyzed under fluorescence microscopy using a Carl Zeiss LSM 5 PASCAL laser scanning microscope.

**NAD(P)H Oxidase Activity**

NTS was washed with ice-cold PBS and homogenized in cold lysate buffer (pH 7.0, 1 mM EGTA, 20 mM KH2PO4, 0.5 µg/mL leupeptin, 10 µg/mL aprtinin, 0.7 µg/mL pepstatin, and 0.5 mM PMSF). The homogenate was centrifuged at 1000g for 20 minutes at 4°C. The pellet was resuspended in a lysis buffer containing protease inhibitors and manually homogenized on ice. NADPH oxidase activity was measured by a luminescence assay in a 20 mmol/L phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 0.25mM dark-adapted lucigenin as the electron acceptor, and 100 µM NADPH. The reaction was started by the addition of 50 µL of homogenate, and luminescence measurements were obtained every 15 s for 60 minutes. Protein content was determined in an aliquot of the
homogenate, and the results were standardized to this measurement. The samples were analyzed using TriStar LB 941 (Berthold Tech. GmbH and Co. KG, Germany).

**Assay of Plasma Catecholamine**

Blood samples were assayed for content of norepinephrine (NE) utilizing enzyme immunoassay for the high-sensitive quantitative determination by Labor Diagnostika Nord-Medicorp Inc.

**Statistical Analysis**

All data are expressed as the mean±SEM. Paired *t*-tests were used to compare BP measurements before and after pretreatment, and one-way analysis of variance (ANOVA) with Scheffe’s post hoc comparison was employed to compare group differences. *P* < 0.05 was considered significant.

**Supplement Result**

**Fructose and Caffeine May Not Influence NO Levels in the Serum**

Serum NO levels in study and control groups are depicted in Figure supplement 1. However, no significant difference was found in serum NO levels in all groups.

**Caffeine May Not Influence A2a-ERK1/2-eNOS Signaling in the NTS**

It has been reported that caffeine is an antagonist of all subtypes of adenosine receptors. In a previous study, we demonstrated that adenosine A2a-ERK1/2-eNOS signaling in the NTS promotes NO production, which is associated with the regulation of BP. Therefore, we determined whether caffeine influences A2a-ERK1/2-eNOS signaling in the NTS. However, immunoblotting analyses revealed no significant changes in the levels of the A1 receptor and A2a receptor in the NTS of all groups. The levels of ERK1/2^{T202/Y204} and eNOS^{S1177} phosphorylation also showed no significant differences following caffeine treatment (Figure S2).

**Caffeine Inhibits Fructose-Induced NADPH Oxidase Activity in the NTS**

Figure S3 shows that NADPH oxidase activity was significantly higher in NTS from fructose fed rats compared with control group rats, and treatment with caffeine significantly prevented the increase in NADPH oxidase activity. Immunoblotting analysis revealed significantly increased gp91 protein levels in the NTS of fructose-fed rats compared to the controls (Figures S4A and S4B, lanes 1 and 2). Following the administration of caffeine, a significant
increase in gp91 protein levels was observed in the fructose-fed rats (Figures S4A and S4B, lanes 2 and 3), whereas the level in the caffeine group was unchanged compared to the control (Figures S4A and S4B, lanes 1 and 4). The results of the immunoblotting analysis demonstrated that fructose increased P67 protein levels in the NTS compared to the controls (Figures S4C and S4D, lanes 1 and 2). As expected, we observed that caffeine inhibited P67 protein levels in the NTS of the fructose group (Figures S4C and S4D, lanes 2 and 3). However, the P67 protein levels were not significantly different between the caffeine and control groups (Figures S4C and S4D, lanes 1 and 4). Immunoblotting analysis demonstrated that fructose could significantly increase P22 protein levels in the NTS (Figures S4E and S4F, lanes 1 and 2). Caffeine pretreatment could attenuate P22 protein levels in the NTS of fructose-fed rats (Figures S4E and S4F, lanes 2 and 3). However, no significant difference in P22 protein levels between the caffeine group and the control group (Figures S4E and S4F, lanes 1 and 4). However, immunoblotting analyses revealed no significant changes in the levels of the P47 protein levels in the NTS of all groups (Figure S4G and S4H).

Reference


Figure S1. Caffeine and Fructose May Not Influence Serum NO. Administration of fructose results in same levels of NO compare with control group. Systemic treatment with caffeine leads to no significantly difference NO levels in the serum in the fructose group, as compared to the fructose controls.
Figure S2. Caffeine May Not Influence A2a-ERK1/2-eNOS Signaling in the NTS. A, Immunoblots revealing that the same levels of Adenosine 1 receptor (A1R) protein are present in the NTS in the fructose group and in the control group (lanes 1 and 2). Caffeine treatment results in no change in the level of A1R protein in the fructose group (lane 3). B, The results of densitometric analyses of A1R levels are shown before and after treatment with fructose or caffeine. The values indicated with bars are the mean±SEM (n=6). C, Immunoblots revealing that the same levels of Adenosine 2 receptor (A2R) protein are present in the NTS in the fructose group and in the control group (lanes 1 and 2). Caffeine treatment results in no change in the level of A2R protein in the fructose group (lane 3). D, Densitometric analyses of A2R levels are shown before and after treatment with fructose or caffeine. The values indicated with bars are the mean±SEM (n=6). E, Immunoblots revealing that the same levels of P-ERK P44/P42 protein are present in the NTS in the fructose group and the control group (lanes 1 and 2). Caffeine treatment results in no change in the level of P-ERK P44/P42 protein in the fructose group (lane 3). F, The results of densitometric analyses of P-ERK P44/P42 levels are shown before and after treatment with fructose or caffeine. The
values indicated with bars are the mean±SEM (n=6). **G**, Immunoblots revealing that same
levels of P-eNOS\textsuperscript{S1177} protein are present in the NTS in the fructose group and the control
group (lanes 1 and 2). Caffeine treatment results in no change in the level of P-eNOS\textsuperscript{S1177}
protein in the fructose group (lane 3). **H**, The results of densitometric analyses of
P-eNOS\textsuperscript{S1177} levels are shown before and after treatment with fructose or caffeine. The values
indicated with bars are the mean±SEM (n=6).

**S3**

![Graph](image)

**Figure S3. Caffeine treatment attenuates NADPH oxidase activity caused by Fructose in the NTS.** Administration of fructose results in high NADPH oxidase activity. Systemic
treatment with caffeine leads to significantly reversed NADPH oxidase activity in the NTS in the fructose group, as compared to the fructose controls. The values indicated with bars are the mean±SEM (n=6). *\(P<0.05\) vs. control and #\(P<0.05\) vs. fructose.
Figure S4. Effect of fructose or caffeine on NADPH oxidase subunits protein levels in the NTS of rats. A. Immunoblot depicting high levels of gp91 protein in the NTS of the fructose group compared to the control group (lanes 1 and 2). Caffeine treatment decreased the level of gp91 protein in the fructose group (lane 3). B, Densitometric analysis of gp91 protein levels before and after treatment with fructose or caffeine. C. Immunoblot depicting high levels of P67 protein in the NTS of the fructose group compared to the control group (lanes 1 and 2). Caffeine treatment decreased the level of P67 protein in the fructose group (lane 3). D, Densitometric analysis of P67 protein levels before and after treatment with fructose or caffeine. E. Immunoblot depicting high levels of P22 protein in the NTS of the fructose group compared to the control group (lanes 1 and 2). Caffeine treatment decreased the level of P22 protein in the fructose group (lane 3). F, Densitometric analysis of P22 protein levels before and after treatment with fructose or caffeine. G, Immunoblots revealing that the same levels of P47 protein are present in the NTS in the fructose group and in the control group (lanes 1 and 2). Caffeine treatment results in no change in the level of P47 protein in the fructose group (lane 3). H, The results of densitometric analyses of P47 levels are shown before and after treatment with fructose or caffeine. The values indicated with bars are the mean±SEM (n=6).