Original Article

Chronic Insulin Infusion Normalizes Blood Pressure and the Gene Expressions of Angiotensin II Type 1 Receptor in Fructose-Fed Rats

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It remains open to debate whether hyperinsulinemia leads to the development of hypertension. We addressed this issue by investigating the effect of chronic insulin infusion on blood pressure and related parameters in hypertensive fructose-fed rats. Rats were given either normal chow or a fructose-rich diet, and insulin or saline was infused through mini-pumps in the same animals for 14 days. The chronic insulin infusion exerted no effect on the blood pressure of the chow-fed rats. Fructose feeding increased the blood pressure and levels of insulin, triglyceride and fatty acid. Insulin infusion augmented the hyperinsulinemia but normalized the blood pressure and plasma lipids. Plasma angiotensin II was elevated in the fructose-fed rats, while insulin infusion left it unchanged. The expression of angiotensin II type 1 receptor (AT1R) mRNA was doubled in both the aorta and epididymal fat of the fructose-fed rats, while that of angiotensin II type 2 receptor (AT2R) was unaltered. Insulin infusion completely rectified the over-expression of the AT1R gene. Our findings indicate that chronic insulin infusion exacerbates hyperinsulinemia while normalizing blood pressure and the gene expressions of AT1R in insulin-resistant fructose-fed rats, suggesting that endogenous hyperinsulinemia caused by insulin resistance is associated with the development of hypertension, whereas exogenous hyperinsulinemia attenuates hypertension probably due to amelioration of insulin resistance. (*Hypertens Res* 2008; 31: 127–133)

***Key Words*: insulin, fructose-fed rat, blood pressure, angiotensin II receptor**

Introduction

Hypertension often develops in individuals with conditions associated with insulin resistance, such as obesity, type 2 diabetes, and metabolic syndrome. The hypertension accompanying insulin resistance is most often explained by hyperinsulinemia compensating for the insulin-resistant conditions. Insulin has been hypothesized to stimulate sympathetic nerve activity, sodium absorption in the renal tubules, and proliferation of smooth muscle cells in vessels (*1*–*3*). It remains unclear, however, whether insulin actually increases blood pressure (BP) *in vivo*. Indeed, patients with insulinoma are usually normotensive (*4*, *5*). Hypertension, on the other hand, has possible links not with hyperinsulinemia, but with insulin resistance. Given that insulin resistance and hyperinsulinemia develop simultaneously, it can be difficult to ascertain the distinct role of hyperinsulinemia in the development of hypertension independently of insulin resistance.

Rats fed a high-fructose diet serve well as an animal model for hypertension associated with insulin resistance and hyperinsulinemia (*6*–*8*). It remains to be determined, however,

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Table 1. General Profiles in Chow-Fed and Fructose-Fed Rats Infused with Saline or Insulin

	Chow	Chow with insulin	Fructose	Fructose with insulin
n	12	8	12	12
SBP (mmHg)	109±4	110±8	119±6*	108±12
Pulse rate (/min)	350±31	333±23	369±14	361±11
Food intake (g/day)	26.5±3.0	28.7±2.6	18.5±3.7*	25.8±5.5
Water intake (g/day)	42.3±3.5	40.6±5.0	42.7±6.5	43.5±4.5
Initial body weight (g)	255±8	260±6	262±6	258±10
Final body weight (g)	410±30	408±20	380±24*	406±28

SBP, systolic blood pressure. *$p<0.05$ chow-fed *vs*. fructose-fed.

whether hyperinsulinemia is a causative factor behind the development of hypertension. Earlier reports have suggested that the renin-angiotensin system plays an important role in the development of hypertension associated with fructose-induced hypertension accompanying insulin resistance/hyperinsulinemia (*9–11*). Giacchetti *et al*. (*12*) reported elevations in the levels of angiotensin II (AII) type 1 receptor (AT1R) mRNA in fructose-fed rats, implicating AT1R mRNA as a possible contributor to fructose-induced hypertension. But it is unclear whether the elevated AT1R gene expression in fructose-fed rats is due to the development of hyperinsulinemia. Insulin has been reported to augment AT1R production *in vitro* (*13*), though it has yet to be determined whether hyperinsulinemia stimulates AT1R production *in vivo*.

We investigated the effects of chronic insulin administration on BP and BP-related factors, such as the gene expressions of AII receptors, in hypertensive fructose-fed rats in order to determine whether further hyperinsulinemia influences hypertension and metabolic derangements induced by fructose feeding.

Methods

Rats

Eight-week-old male Wistar rats (Charles River Japan, Tokyo, Japan) were divided into two groups and fed either standard rat chow containing 60% vegetable starch, 5% fat, and 24% protein (Oriental Yeast Co., Tokyo, Japan) or a fructose-rich chow containing 60% fructose, 5% fat, and 20% protein (Oriental Yeast Co.) for 21 days. Both diets contained 0.1% NaCl (w/w). Saline (0.9% NaCl) was used for both the control (saline anole) and insulin (solved with saline) infusions. During the last 14-day feeding period, the chow-fed and fructose-fed rats were each divided into subgroups and continuously infused with either human insulin (Novolin R, 6 U/24 h) solved with saline or saline vehicle alone at a rate of 4.5 μL/h by an osmotic mini-pump (Alzet Model 2ML2; Alza Corp., Palo Alto, USA) implanted subcutaneously. All rats were kept in individual cages on a rotating 12-h light–dark cycle with free access to food and water. The animals were fasted from 9:00 AM on the day of the experiment up to the commencement of the experiment at 2:00 PM. Drinking water remained available. All procedures were approved by the Institutional Animal Care and Use Committee of Showa University according to the Guidelines for the Care and Use of Laboratory Animals.

BP and Heart Rate Measurement

The systolic BP (SBP) and pulse rate were recorded in completely conscious rats using indirect tail-cuff equipment (BP Monitor MK-1030; Muromachi Kikai Co., Ltd., Tokyo, Japan) as described previously (*14*). The BP and pulse rate were each measured 5 times after pre-warming the rats on a 37°C plate for 20 min. The mean values were taken as the individual BP and pulse rate of each rat.

Gene Expression of AT1R and AII Type 2 Receptor in the Aorta and Adipose Tissue

Total RNA was extracted from the aortic tissue and epididymal fat pads using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription was carried out using 800 ng of total RNA with the ReverTra Dash™ RT-PCR Kit (Toyobo, Osaka, Japan) and Oligo(dT)20 primer. The following rat oligonucleotide primers were used: for AT1R, 5′-CCAGAAAAACAAAATGGACC-3′ (forward) and 5′-TACATTTCGGTGGATGACAG-3′ (reverse); and for β-actin, 5′-ACTGGCATTGTGATGGACTC-3′ (forward) and 5′-GTGGTGGTGAAGCTGTAGCC-3′ (reverse). Polymerase chain reaction (PCR) was performed for 30 cycles (98°C for 10 s, 60°C for 30 s, 74°C for 30 s, and a final elongation step of 7 min at 72°C) for AT1R and β-actin. The rat oligonucleotide primers for AII type 2 receptor (AT2R) were as follows: 5′-AAGAGTGTAAGGATTGGGAG-3′ (forward) and 5′-TTCAGGGTCAGAAAAGAACC-3′ (reverse). PCR was performed for 32 cycles (94°C for 30 s, 56°C for 30 s, 74°C for 60 s, and a final elongation step of 7 min at 72°C) for AT2R and β-actin. The reverse transcription (RT)–PCR products were resolved electrophoretically on 2% agarose gel, and the gel was stained with ethidium bromide and photographed under UV light. The extent of target gene

Table 2. Plasma Concentrations in Chow-Fed and Fructose-Fed Rats Infused with Saline or Insulin

	Chow	Chow with insulin	Fructose	Fructose with insulin
n	12	8	12	12
Total cholesterol (mg/dL)	82±12	94±12	107±28*	78±13†
Triglyceride (mg/dL)	92±43	63±24†	332±150**	157±97
Glucose (mg/dL)	182±20	154±22†	189±26	156±47†
Free fatty acid (mEq/L)	1.73±0.37	0.94±0.25‡	2.78±0.90**	1.41±0.73‡
Rat insulin (ng/mL)	2.72±1.12	NA	4.47±2.24*	NA
Rat C-peptide (ng/mL)	5.56±1.25	NA	7.17±1.49*	NA
Human insulin (μU/mL)	1.40±3.40	42.06±17.37‡	1.68±2.66	57.92±44.72‡
Angiotensin II (pg/mL)	150±21	187±81	473±125**	583±235

*$p<0.05$, **$p<0.01$ chow-fed *vs*. fructose-fed. †$p<0.05$, ‡$p<0.01$ saline infusion *vs*. insulin infusion. NA, not available.

expression was given as a relative unit which was defined as the quotient of target gene expression *vs*. β-actin gene expression.

Biochemical Assays

Plasma insulin (Morinaga, Yokohama, Japan), and C-peptide (Wako Pure Chemical Industries, Osaka, Japan) were each measured by an ELISA kit for rats. Human insulin (Eiken Kagaku, Tokyo, Japan) was measured by a 2-step sandwich enzyme-immunoassay method. The plasma AII level was measured by radioimmunoassay (SRL, Tokyo, Japan). The plasma levels of glucose, free fatty acids (FFA), triglyceride, and total cholesterol were measured in spectrophotometers with standard commercial kits (Wako Pure Chemical Industries, Osaka, Japan).

Statistical Analysis

Results are expressed as the means±SD. One-way ANOVA and the independent *t*-test were used to evaluate differences of means between different groups. Correlations between two parameters were analyzed using Pearson's simple correlation analysis. Statistical significance was accepted at $p<0.05$.

Results

General Profiles of Chow-Fed Rats and Fructose-Fed Rats with and without Insulin Infusion

The SBP was slightly but significantly higher in the fructose-fed rats than in the chow-fed rats (Table 1). Insulin infusion completely rectified the elevated SBP in the fructose-fed group. Insulin infusion left the SBP unchanged in the chow-fed group. Neither the fructose feeding nor insulin infusion exerted any apparent influence on the pulse rate. Food intake was comparable among the chow-fed, insulin-infused chow-fed, and insulin-infused fructose-fed rats, whereas the food intake and the body weight were somewhat lower in the fructose-fed rats than in the other groups.

Plasma Measurements in Chow-Fed Rats and Fructose-Fed Rats with and without Insulin Infusion

Table 2 shows the plasma lipids, glucose, and various hormones in chow-fed and fructose-fed rats with and without insulin infusion. Insulin infusion significantly decreased plasma FFA and triglyceride levels without affecting total cholesterol in the chow-fed rats. The fructose-fed rats had markedly higher plasma levels of triglyceride and FFA compared to the chow-fed rats, while their total cholesterol level was only slightly higher. Insulin infusion halved the triglyceride level and completely restored the elevated levels of cholesterol and FFA. Plasma glucose levels were comparable between the chow-fed and fructose-fed rats and significantly decreased in both groups in response to the insulin infusion. The plasma levels of rat insulin and rat C-peptide were significantly elevated in the fructose-fed rats. Insulin infusion markedly increased the level of human insulin from 1.4 to 42.1 μU/mL in the chow-fed rats and from 1.7 to 57.9 μU/mL in the fructose-fed rats. The plasma AII level was significantly higher in the fructose-fed rats than in the chow-fed rats. Insulin infusion left the plasma AII level unchanged in both the chow-fed and fructose-fed groups.

Expression of AT1R and AT2R mRNA in the Aorta

Figure 1 depicts the expression of AT1R mRNA (expressed by the ratio to β-actin mRNA) in the aortas of the chow-fed and fructose-fed rats with and without insulin infusion (top). Insulin infusion had no effect on the AT1R mRNA level in the chow-fed rats. The level of AT1R mRNA in the aorta was doubled in the fructose-fed rats, whereas the insulin infusion remarkably suppressed it to below the level measured in the chow-fed rats. In contrast, the AT2R mRNA levels in the aorta were left unchanged by fructose feeding or insulin treatment (Fig. 1, bottom).

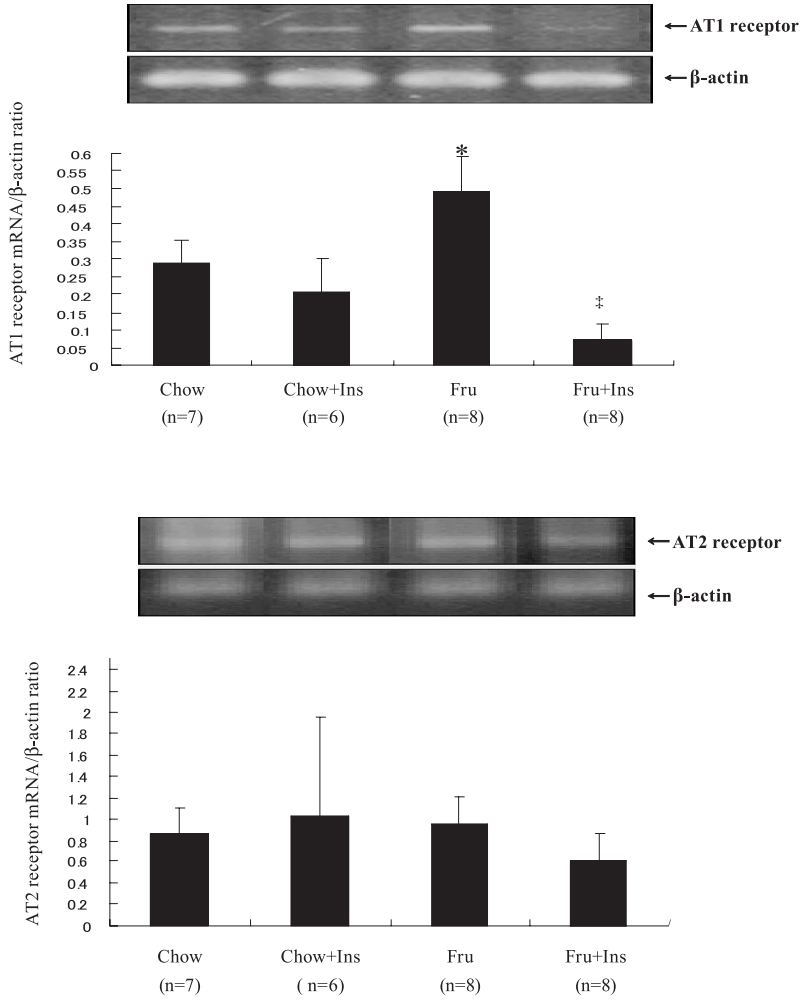

Fig. 1. *Angiotensin II type 1 (AT1) receptor mRNA/β-actin ratio (top) and angiotensin II type 2 (AT2) receptor mRNA/β-actin ratio (bottom) in the aorta. Chow, chow-fed rats; Ins, insulin infused; Fru, fructose-fed rats. *$p < 0.05$ chow vs. fructose; ‡$p < 0.01$ saline infusion vs. insulin infusion.*

Expression of AT1R and AT2R mRNA in the Epididymal Fat Pads

Figure 2 depicts the expression of AT1R mRNA (expressed as the ratio to β-actin mRNA) in the epididymal fat pads of the chow-fed and fructose-fed rats with and without insulin infusion (top). Insulin infusion left the expression of AT1R mRNA unchanged in the chow-fed rats. As found in the aorta, the level of AT1R mRNA in the epididymal fat was doubled in the epididymal fat pads of the fructose-fed rats, whereas the insulin infusion returned it to normal. Once again, the AT2R mRNA levels of the adipose tissue were left unchanged by fructose feeding or insulin treatment (Fig. 2, bottom).

Discussion

Several papers have demonstrated the effects of insulin administration on BP in normal rats, but the results have often been conflicting. Hsieh (*15*) reported that acute insulin infusion for several hours significantly increased mean arterial pressure in chow-fed rats. However, it is likely that acute insulin infusion leads to a sub-physiological hypoglycemia *in vivo* and stimulates the secretion of the counter-regulatory hormones which increase BP. Unlike the results following acute insulin infusion, we did not observe any changes of BP in rats chronically infused with insulin. Similar to our result, several papers (*16*, *17*) showed no evidence of a BP elevation in chow-fed rats treated with insulin over a long time course. In contrast, Juan *et al*. (*18*) and Fang *et al*. (*19*) reported elevations in BP over the course of long-term insulin infusion.

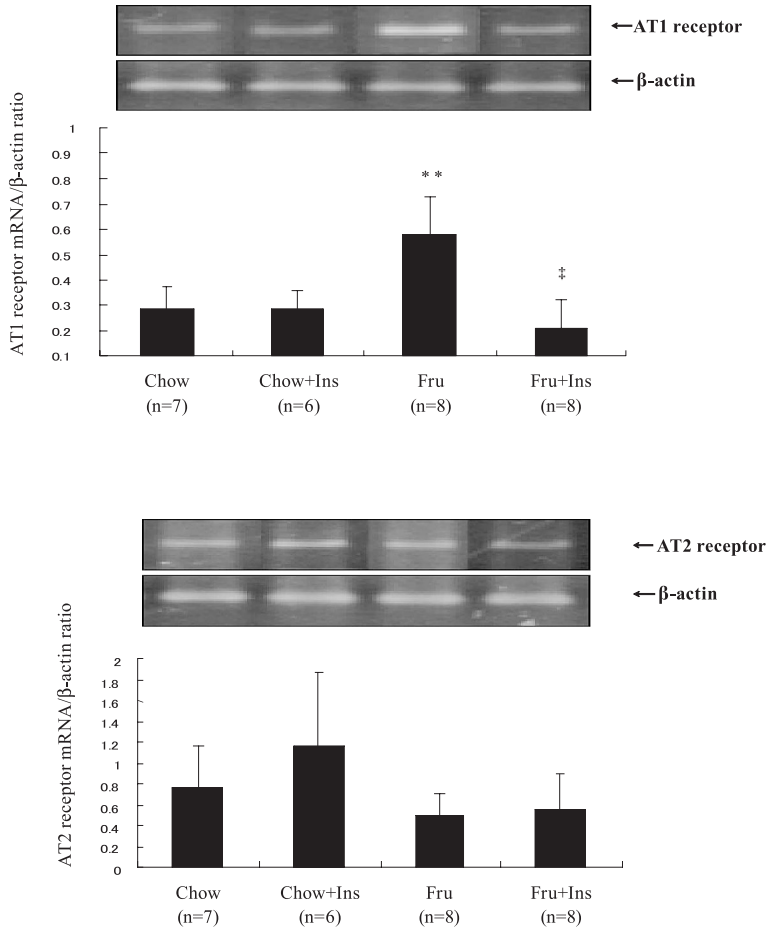

Fig. 2. *Angiotensin II type 1 (AT1) receptor mRNA/β-actin ratio (top) and angiotensin II type 2 (AT2) receptor mRNA/β-actin ratio (bottom) in the epididymal fat.* ***p* < 0.01 *chow vs. fructose;* ‡*p* < 0.01 *saline infusion vs. insulin infusion.*

The elevation in BP was observed after 14 days in the former study (*18*) and from 28 days in the latter (*19*). Our group only examined the BP once, on day 14 after the commencement of insulin infusion. In retrospect, it seems that infusion over this limited period might have been insufficient to induce any BP elevation. We must note, however, that the insulin resistance in both studies emerged after a long period of insulin infusion, and further, that the BP never rose above normal before the insulin resistance developed. These findings suggest that the hypertension results not predominately from the hyperinsulinemia, but from the insulin resistance. Several lines of evidence suggest that insulin inherently relaxes and dilates the arterial wall (*20–22*). Insulin resistance, on the other hand, has been established to impede the insulin-mediated vasodilatation. To cite one example, we know that the activity of endothelial nitric oxide synthase, a limiting enzyme for nitric oxide generation, is increased by insulin (*23*, *24*) and decreased in an insulin-resistant state (*25*).

The BP of the fructose-fed rats was only 10 mmHg higher than that of the chow-fed rats. We speculate that this small increment of BP might have been attributable to a reduction in salt intake resulting from the decrease in food consumed. Unlike the Sprague-Dawley rat, a strain whose BP consistently rises in response to fructose feeding, the Wistar rat requires salt in the diet to develop fructose-induced hypertension (*26*, *27*). Thus, the modest BP elevation in our experiments may be attributable to two factors in combination: the low salt-intake and the strain of rats used. The food intake did not decrease in the fructose-fed animals infused with insulin. Thus, the additional hyperinsulinemia does not seem to elevate the BP even when the salt intake is increased.

Chronic insulin infusion severely exacerbated the degree of hyperinsulinemia in fructose-fed rats, though it exerted no effect in increasing the BP. To the contrary, the BP was completely normalized. In parallel to this finding, we observed that chronic insulin infusion markedly suppressed the overexpression of the AT1R gene in the aortas of the fructose-fed rats. The same process was also observed in the adipose tissue. Nickenig *et al*. reported that insulin augmented AT1R production in vascular smooth muscle cells (*13*), though it has yet to be determined whether hyperinsulinemia stimulates AT1R production *in vivo*. The conflicting results may in part

involve the presence or absence of insulin resistance (*i.e.*, fructose-fed rats *vs*. normal rat cells). The levels of AT1R mRNA are reported to be significantly higher in fat obtained from fructose-fed animals than fat obtained from control animals (*9*, *12*, *28*). Thus, it is apparent that endogenous hyperinsulinemia due to insulin resistance dose not up-regulate AT1R gene expression. Nickenig *et al*. (*13*) used an extremely high concentration of insulin (100 nmol/L), which might down-regulate insulin receptors and led to the development of insulin resistance in the cells.

We found that the plasma AII level rose in fructose-fed rats, duplicating a finding from an earlier report by Iyer *et al*. (*28*). BP was decreased by insulin treatment in spite of increased AII levels in fructose-fed rats, suggesting that AT1R plays a more crucial role in regulating BP than the circulating AII level. We speculate that an excess amount of exogenous insulin overcomes fructose-induced insulin resistance, which attenuates hypertension by the suppression of AT1R gene expression and the revealing the inherent action of insulin on vasodilatation. The mechanisms of fructose-induced hypertension in the rat are multi-factorial. The sympathetic nerve system, endothelin-1 nitric oxide, oxidative stress, and kallikrein/kinin systems were proposed to be involved in the pathogenesis of fructose-induced hypertension, and it is possible that insulin treatment affects these factors. Therefore, we may have to pay attention to these factors in addition to the renin-angiotensin system and insulin resistance.

Several studies have reported that endogenous hyperinsulinemia is closely associated with the development of hypertension in fructose-fed rats. In the study by Verma *et al*. (*29*), the suppression of hyperinsulinemia by neonatal sympathectomy completely prevented the development of fructose-induced hypertension. In a study by Reaven *et al*. (*30*), the suppression of hyperinsulinemia by somatostatin administration completely prevented the development of fructose-induced hypertension. Thus, endogenous hyperinsulinemia could be a causative factor for the development of hypertension in this animal model of insulin resistance.

In conclusion, chronic insulin infusion induced hyperinsulinemia but normalized BP and the gene expressions of AT1R in fructose-fed rats, an animal model of hypertension associated with hyperinsulinemia/insulin resistance, suggesting that endogenous hyperinsulinemia caused by insulin resistance is associated with the development of hypertension, whereas exogenous hyperinsulinemia attenuates hypertension probably due to amelioration of insulin resistance.

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