Effect of Administration of Insulin on Streptozotocin-Induced Diabetic Hypertension in Rat

Shanwan Chen, Christina M. Yuan, Francis J. Haddy, Motilal B. Pamnani

Abstract We have reported that streptozotocin-induced insulin-dependent diabetes mellitus in 25% reduced renal mass rats is associated with low-renin, volume-expanded hypertension and that the development of the hypertension can be prevented with insulin. In this study we examined the effect of insulin after the animals had developed sustained hypertension. Normotensive 25% reduced renal mass rats were treated with streptozotocin and, as expected, developed insulin-dependent diabetes mellitus and hypertension. After 4 weeks of sustained hypertension, neutral protamine Hagedorn insulin (6 to 8 IU/d) was administered subcutaneously for 4 weeks. As expected, insulin treatment decreased plasma glucose and increased body weight gain relative to untreated diabetic rats. On the other hand, insulin treatment did not reverse the hypertension and albuminuria. It also did not normalize extracellular fluid volume and plasma renin activity. Furthermore, insulin treatment did not reverse the increase in plasma Na⁺,K⁺-ATPase inhibitory activity (determined by both radio-immunoreassay and bioassay) and the inhibition of myocardial microsomal Na⁺,K⁺-ATPase activity observed in the untreated diabetic hypertensive rats. 5'-Nucleotidase, a membrane marker, was not different between insulin-treated and untreated diabetic rats. These results show that insulin, given as here described, does not reverse the insulin-dependent diabetes mellitus hypertension in 25% reduced renal mass rats once it is established, perhaps because it does not reverse the albuminuria, volume expansion, increase in endogenous digitalis-like substance, and inhibition of cardiovascular muscle cell Na⁺,K⁺-ATPase activity. (Hypertension. 1994;23[part 2]:1046-1050.)

Key Words • insulin • digitalis • hypertension, experimental • diabetes mellitus • streptozotocin

The role of insulin in blood pressure regulation is complicated. Insulin increases renal sodium absorption and sympathetic nerve activity, both of which tend to elevate arterial pressure. On the other hand, insulin stimulates Na⁺,K⁺ pump activity, thereby causing electrogenic hyperpolarization of the vascular muscle cell and vasodilation. Clinically, insulin treatment has no significant effect on insulin-dependent diabetes mellitus (IDDM) hypertension.1 Chronic administration of insulin in experimental IDDM animals has been shown to increase,2 decrease,3 and have no effect on blood pressure.

We have previously shown that hypertension regularly develops when rats with 25% reduced renal mass (RRM) are treated with streptozotocin and that this hypertension is associated with albuminuria, increased extracellular fluid volume (ECFV), and the appearance in plasma of an Na⁺,K⁺-ATPase inhibitor and that the albuminuria, volume expansion, appearance of the Na⁺,K⁺-ATPase inhibitor, and hypertension can be prevented by treatment with insulin at the time the IDDM is induced, ie, if the insulin treatment is started before the development of hypertension.7 In the latter study, neutral protamine Hagedorn (NPH) insulin treatment (initially 4 IU/d for 2 to 3 days and then 6 IU/d) immediately after streptozotocin injection prevented the hypertension and all the other changes, even though blood glucose was not completely normalized.

There were three groups: (1) 25% RRM alone, (2) 25% RRM plus streptozotocin diabetes, and (3) 25% RRM plus streptozotocin plus insulin. Blood pressure rose in the untreated group 2 but not in the insulin-treated group 3 (blood pressure was not different from that in the control group 1 animals). Body weight increased at a normal rate in the treated group 3 but not in the untreated group 2. Albuminuria, which occurred in the untreated group, did not occur with insulin treatment. Left ventricular microsomal Na⁺,K⁺-ATPase activity, which was suppressed in the untreated group, was normal in the insulin-treated group. The same was the case for Na⁺,K⁺-ATPase activity of right ventricular microsomes. The Na⁺,K⁺-ATPase inhibitory activity in the plasma remained normal with insulin treatment, as did plasma digoxin-like immunoreactive factor, plasma renin activity (PRA), ECFV, left ventricular weight, and kidney weight, even though blood glucose was not completely normalized (203 mg/dL).

In the present study we examined the effect of essentially the same insulin treatment on established hypertension in streptozotocin-induced IDDM rats with 25% RRM, ie, the hypertension was allowed to develop before insulin was given. This posttreatment failed to ameliorate the hypertension and most of the changes associated with it.

Methods

Experimental Animals

Male Wistar rats weighing 250 to 280 g (Charles River) with documented normotension underwent 25% surgical RRM. Briefly, the lower poles of both kidneys (each 12.5% of total renal mass) were removed through a midline abdominal
incision. This was achieved by encircling the lower pole of each kidney with a loop of No. 0000 silk suture and then tightening the loop. This method both cut the tissue and tied off the vessels. After 2 weeks of surgical recovery and documented normotension, when body weight was 350 to 390 g, the animals were entered into the study.

IDDM was induced by the jugular vein injection of 65 mg/kg body wt streptozotocin (Sigma Chemical Co) in citrate buffer saline, pH 4.5. After 4 weeks of sustained diabetes and hypertension, NPH insulin (Eli Lilly & Co) (6 to 8 IU) was subcutaneously administered each day. The final injection was given the day before the animals were killed. Three groups of rats were studied: (1) 25% RRM rats with streptozotocin-induced diabetic hypertension treated with insulin (25-DM-In), (2) 25% RRM rats with streptozotocin-induced IDDM and hypertension (25-DM), and (3) 25% RRM rats receiving vehicle (citrate saline) (25-V). All rats were given tap water and a standard rat chow diet (Bioservices Inc) ad libitum.

Membrane Preparation

Microsomal fractions were prepared from ventricles and renal medullas as described previously. Briefly, the right and left ventricles and renal outer medulla were each minced with a scalpel and homogenized with a motor-driven polytetrafluoroethylene pestle in 10 mL deoxycholate solution at pH 6.8. The homogenate was centrifuged (SM-24 rotor) at 11000g for 10 minutes to remove the nuclear fraction and at 12 300g for 20 minutes to remove the mitochondrial fraction. The supernatant was then centrifuged at 35 600g for 1.5 hours to obtain the microsomal fraction. The microsomal fraction was treated with 1 mol/L NaI solution and recovered in 10 mmol/L imidazole-HCl (pH 7.4) solution.

Enzyme Activity Assays

Membrane ATPase activity was assayed by measuring the amount of inorganic phosphate liberated from ATP (Tris-ATP, Sigma) by a 1-hour incubation at 37°C. The assay medium for total ATPase activity contained in 2 mL (mmol/L) NaCl 120, KCl 10, Tris-HCl 40 (pH 7.5 at 37°C), Tris-ATP 2, MgCl₂ 2.5, and EGTA 0.5. Mg²⁺-ATPase activity was assayed under the same conditions except that KC1 was omitted and documented pentobarbital anesthesia, blood samples were collected, and the hearts and kidneys were removed for membrane preparation.

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Radioimmunoassay for Plasma Digoxin-Like Immunoreactive Factor

Plasma from experimental and control rats was extracted as described previously. One millilitre of plasma and 2 mL ethanol were mixed and boiled at 95°C for 5 minutes. The supernatant was centrifuged at 35 600g for 20 minutes, dried and dissolved in 1 mL deionized water, and then eluted using C18 cartridges (Millipore). The C18 columns were prewashed with 5 mL methanol and then with 10 mL water. After extraction of the deproteinized plasma samples, the columns were washed with 10 mL water and then eluted with 4 mL methanol. The methanol fractions were dried and reconstituted with deionized water. The digoxin-like immunoreactive factor was estimated using a commercially available digoxin radioimmunoassay (IncStar). A direct competitive inhibition radioimmunoassay was performed using plasma digoxin [125I] and an antiserum to digoxin. The antiserum was generated against digoxin-salmine-saponin conjugate in rabbits. The results were expressed as the mean ± SEM of triplicate determinations.

Plasma Bioassay for Digitalis-Like Substance

Plasma from experimental and control rats was bioassayed for digitalis-like substance using a canine kidney Na⁺,K⁺-ATPase preparation as described previously. Briefly, 1 mL plasma and 2 mL ethanol were mixed and boiled at 95°C for 5 minutes. The supernatant was dried and dissolved in 1 mL deionized water and then applied to C18 cartridges. The columns were washed with 8 mL water and then eluted with 2.5 mL of 50% ethanol. The ethanol fraction was dried and reconstituted with deionized water. Canine microsomal ATPase was freshly prepared from normal dog kidney as described previously. The final medium for ATPase activities was the same as described above, except that 0.2 μmol/L ouabain was added for Mg⁺⁺-ATPase activity. Percent inhibition was calculated as (1—Ap/Ao)×100%, where Ap and Ao are Na⁺,K⁺-ATPase activity with or without plasma, respectively.

Other Measurements

Just before animals were killed, blood pressure and heart rate were recorded with animals under pentobarbital (50 mg/kg) anesthesia through a PE-50 carotid artery catheter using a pressure transducer (model P23Db, Gould Statham). In some animals, ECFV and PRA were determined 8 weeks after streptozotocin (4 weeks after insulin treatment) or vehicle injection. ECFV was measured using sodium thiocyanate as described previously. PRA was measured using a commercially available kit (IncStar). Urinary albumin excretion was determined in a 24-hour urine collection using an enzyme-linked immunooassay. Plasma glucose was measured by a glucose analyzer (Beckman Instruments).

Data Analysis

All data are expressed as mean±SEM. Statistical significance was determined using a two-tailed t test for comparing the means of independent samples. ANOVA was used to detect possible significant differences among groups. For repeated measurements in a given group, ANOVA was used followed by Duncan’s multiple range test to determine the significance of the F ratio. A value of P<.05 was considered significant.

Results

After intravenous injection of streptozotocin, all 25% RRM rats developed diabetes (as indicated by hyperglycemia [see the Table]) and an increase in blood pressure (Fig 1A). This was temporally correlated with albuminuria (Fig 1B). The 25-V rats remained normotensive and normoalbuminuric throughout (Fig 1A and 1B). After insulin treatment the 25-DM-In rats had lower blood glucose levels and plasma osmolality than 25-DM rats (Table). The 25-DM-In rats also gained weight, whereas the 25-DM rats did not during the insulin treatment (Fig 1C). However, there were no differences in blood pressure and urinary albumin excretion between insulin-treated and untreated diabetic rats (Fig 1A and 1B). Heart rate was significantly increased in 25-DM-In rats relative to 25-DM rats (Table). Left ventricular weight increased in both groups of diabetic rats (25-DM) relative to 25-V rats (Table). No differences were found in right ventricular weight among the three groups (Table). Kidney weight decreased in 25-DM-In rats relative to 25-DM rats, but both diabetic groups showed significant renal hypertrophy relative to 25-V rats (Table). The 25-DM-In rats had lower PRA and higher ECFV values than 25-V rats, but the values were not different from those in 25-DM rats.

The myocardial microsomal Na⁺,K⁺-ATPase activity of the left and right ventricles was significantly decreased in both 25-DM-In and 25-DM rats relative to 25-V rats. The values in 25-DM-In and 25-DM rats
Extracellular Fluid Volume, Plasma Renin Activity, Mean Blood Pressure, Heart Rate, and Organ Weights in 25% Reduced Renal Mass Rats Treated with Streptozotocin and Insulin

<table>
<thead>
<tr>
<th>Experimental Rats</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mg/dL</th>
<th>Plasma Osmolality, Osm/kg</th>
<th>ECFV, mL/100 g body wt</th>
<th>PRA, (ng Ang I/mL)/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-DM-ln (n=10)</td>
<td>455±13*</td>
<td>218±8.8†</td>
<td>309±2.8*</td>
<td>214±0.49†</td>
<td>9.89±2.2†</td>
</tr>
<tr>
<td>25-DM (n=9)</td>
<td>357±12†</td>
<td>506±10.5‡</td>
<td>328±2.4‡</td>
<td>23.1±1.3‡</td>
<td>7.41±1.4‡</td>
</tr>
<tr>
<td>25-V (n=9)</td>
<td>476±12</td>
<td>153±6.3</td>
<td>301±1.4</td>
<td>16.8±0.27</td>
<td>20.1±3.1</td>
</tr>
</tbody>
</table>

ECFV indicates extracellular fluid volume; PRA, plasma renin activity; Ang I, angiotensin I; MBP, mean blood pressure; LV, left ventricle; RV, right ventricle; 25-DM-ln, 25% reduced renal mass rats treated with streptozotocin and insulin; 25-DM, with streptozotocin alone; and 25-V, with vehicle. Values are mean±SEM.

*P<.05 25-DM-ln vs 25-DM rats.
†P<.05 25-DM-ln vs 25-V rats.
‡P<.05 25-DM vs 25-V rats.

were not significantly different (Fig 2A and 2B). The myocardial Mg$^{2+}$-ATPase and $5'$-nucleotidase activities were not different in the three groups of animals (Fig 2A and 2B). Renal medullary Na$^+$,K$^+$-ATPase activity of both diabetic groups was significantly greater than that of 25-V rats (Fig 2C). However, the renal Na$^+$,K$^+$-ATPase activity of 25-DM-ln rats was significantly lower than that in 25-DM rats (Fig 2C). Mg$^{2+}$-ATPase and $5'$-nucleotidase activities of kidney were not different among the three rat groups. Plasma digoxin-like immunoreactive factor (measured by radioimmunoassay) and digitalis-like substance (determined by bioassay) were elevated in both diabetic rat groups relative to 25-V rats, but they did not differ in the two groups of diabetic animals (Fig 3A and 3B).

**Discussion**

These studies show that in this model of diabetic hypertension insulin in the amount given does not affect blood pressure once the hypertension is established. This contrasts with our previous study in which early treatment with essentially the same amount of insulin prevented the development of hypertension. How do we explain the difference? In our previous study early treatment prevented the appearance of albuminuria, increased ECFV, depressed PRA, increased plasma Na$^+$,K$^+$-ATPase inhibitor, and depressed myocardial Na$^+$,K$^+$-ATPase activity. In the current study insulin had no effect on these parameters; ie, once the albuminuria, increased ECFV, decreased PRA, increased plasma Na$^+$,K$^+$-ATPase inhibitor, and decreased myocardial Na$^+$,K$^+$-ATPase activity had appeared, they were not affected by the insulin treatment. This was the case despite the fact that insulin treatment partly corrected plasma and urine glucose levels and increased body weight.

One possibility is that nephropathy, once established, is not reversed by insulin. Albuminuria is thought to be an index of diabetic nephropathy. Certainly the albuminuria did not improve with insulin treatment. The ability to excrete sodium and water might not have increased either. This would account for the sustained increase in ECFV, which is thought to be the stimulus for release of digitalis-like substance. The insulin treatment did partly reverse the renal hypertrophy and the increased renal Na$^+$,K$^+$-ATPase activity (thought to result from increased single-nephron glomerular filtration rate and sodium load) seen in this model. The latter would be expected to decrease tubular sodium reabsorption and decrease renal blood flow, thus decreasing ECFV.

Fig 1. Line graphs show systolic blood pressure (A), urinary albumin excretion (B), and body weight (C) in 25% reduced renal mass rats treated with streptozotocin and insulin (25-DM-ln), streptozotocin alone (25-DM), and vehicle (25-V). STZ indicates streptozotocin; lnj, injection.
Continued

<table>
<thead>
<tr>
<th>MBP, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>LV, g/100 g body wt</th>
<th>RV, g/100 g body wt</th>
<th>Kidney, g/100 g body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>137±5.7†</td>
<td>330±13†</td>
<td>0.220±0.002†</td>
<td>0.05±0.001</td>
<td>0.68±0.08†</td>
</tr>
<tr>
<td>132±4.6†</td>
<td>284±10†</td>
<td>0.228±0.004†</td>
<td>0.05±0.004</td>
<td>0.91±0.16†</td>
</tr>
<tr>
<td>109±3.6</td>
<td>383±12</td>
<td>0.186±0.001</td>
<td>0.05±0.001</td>
<td>0.54±0.05</td>
</tr>
</tbody>
</table>

Increased plasma digitalis-like substance was demonstrated by both radioimmunoassay and bioassay. Myocardial Na⁺,K⁺-ATPase activity decreased, and the extent of the decrease was not influenced by insulin (even though insulin has the propensity to increase Na⁺,K⁺-ATPase activity). The decrease in myocardial Na⁺,K⁺-ATPase activity cannot be secondary to changed microsomal distribution caused by ventricular hypertrophy; the left and right ventricles had the same changes in Na⁺,K⁺-ATPase activity even though the right ventricle was not hypertrophied. Furthermore,
5'-nucleotidase, a plasma membrane marker, was not different among the three groups. Inhibition of Na⁺,K⁺-ATPase appears to increase blood vessel and heart contractility and raise blood pressure.13-15

Insulin partly corrected the bradycardia seen in the 25-DM rats. This might reflect increased sympathetic nervous system action. Insulin also may have increased the sensitivity of the heart to catecholamines.16

In summary, even though early insulin treatment prevents the development of hypertension in our model of diabetes hypertension, it fails to ameliorate the hypertension once established. Only one insulin regimen was studied; others should also be explored.

References
Effect of administration of insulin on streptozotocin-induced diabetic hypertension in rat.
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Hypertension. 1994;23:1046-1050
doi: 10.1161/01.HYP.23.6.1046
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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