Scientific Contributions

Human Insulin-Mediated Enhancement of Vascular β-Adrenergic Responsiveness

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Abstract Insulin may play an important role in the physiological and/or pathophysiological regulation of the cardiovascular system. Insulin augments sympathetic activity,1 increases renal sodium retention,2 and increases forearm blood flow.3 Furthermore, hyperinsulinemia and insulin resistance have been associated with elevated blood pressure.4,5 However, a causal relation has yet to be established.

Recent studies have focused on the role of insulin as an endogenous vasodilator. In humans, insulin increases blood flow and reduces vascular resistance despite augmentation of sympathetic activity.1 Further insulin-mediated increases in blood flow were suggested to occur via a β-adrenergic mechanism (ie, blocked by β-adrenergic receptor antagonists) in both canine6 and human3 studies. However, the mechanism for this effect remains unexplained. Therefore, to determine whether insulin might have a role in regulating vascular responsiveness through alterations in adrenergic responsiveness, we examined the effect of insulin on tension development in isolated rat thoracic aortic ring segments. Data indicated that insulin enhances β-adrenergic responsiveness in vessels from normotensive animals, that this effect is endothelium dependent, and that this enhancement is lost in spontaneously hypertensive rats (SHR).

Methods

Drugs

Phenylephrine hydrochloride, (+)-isoproterenol hydrochloride, sodium nitroprusside, and acetylcholine chloride were obtained from Sigma Chemical Co. Human insulin was obtained from Eli Lilly & Co. All standard laboratory chemicals were obtained from BDH.

Animals

Male SHR and normotensive Wistar rats (200 to 300 g, Charles River) were used throughout. The rats were cared for in accordance with Canadian Council on Animal Care guidelines and housed under a 12-hour light/dark cycle, with free access to standard laboratory rat chow and drinking water. Indirect tail-cuff measurements of systolic blood pressure were obtained in lightly anesthetized rats (2% halothane in a mixture of 50% nitrous oxide and 50% oxygen) using a Harvard rat tail blood pressure monitor (Ealing).

Assessment of Tension in Aortic Ring Segments

Assessment of tension in aortic ring segments was performed according to our previously published methods.7 Briefly, rats were anesthetized with 5% halothane in a mixture of 50% nitrous oxide in oxygen, the chest wall was opened, and a portion of thoracic aorta was removed quickly to a Petri dish containing ice-cold Krebs physiological salt solution (KFSS) of the following composition (mmol/L): NaCl 118.0, NaHCO3 25.0, d-glucose 11.1, KCl 4.72, CaCl2 2.56, NaH2PO4 1.13, and MgCl2 1.12. (–)Ascorbic acid (0.114 mmol/L) and disodium EDTA (0.0297 mmol/L) were added to stabilize test drugs.

Blood and connective tissue were removed carefully, and four ring segments (2 to 3 mm wide) from each aorta were cut and suspended in KFSS under optimal tension (0.75 to 1.0 g, determined in preliminary experiments; data not shown) in individually double-walled organ baths maintained at 37°C and gassed with 5% CO2 and 95% O2. The rings were equilibrated for 60 minutes, during which the KFSS was changed three times and the tension adjusted as necessary. Tension generated was measured using isometric force-displacement transducers (FT03, Grass Instrument Co) and displayed on a polygraph (Grass model 7B).

After the equilibration period, rings were maximally preconstricted with phenylephrine (final bath concentration, 3 μmol/L) and allowed to reach a plateau that remained stable for more than 30 minutes (mean tension, 0.22±0.03 g/mg tissue, n=11 rings). Baseline relaxation response was assessed...
by a single addition of isoproterenol (final bath concentration, 10 μmol/L) or in some experiments sodium nitroprusside (10 nmol/mL) to phenylephrine-preconstricted rings. The isoproterenol- or nitroprusside-mediated relaxation was followed over 20 minutes. After washout of phenylephrine and isoproterenol (or sodium nitroprusside), two of four rings were incubated in the presence of increasing concentrations of human insulin (0.01, 0.1, 1.0, and 10.0 nmol/mL). Insulin was added concurrent with the addition of phenylephrine. Isoproterenol- or nitroprusside-mediated relaxation in phenylephrine-preconstricted rings in the presence of insulin was assessed as above. In each experiment, two of four rings were assessed after sequential cycles of constriction and relaxation without insulin and thus served as temporal controls.

In experiments assessing the role of the endothelium in insulin-mediated effects, gentle rubbing and flushing with distilled water was used to denude the blood vessels of endothelium. The absence of functional endothelium was inferred by the loss of acetylcholine-mediated relaxation in phenylephrine-preconstricted rings.

**Determination of Isoproterenol Concentrations in Organ Bath Samples**

To determine the stability of isoproterenol in the organ bath, samples were taken at 1, 2, 4, 6, 8, 10, and 20 minutes after addition of isoproterenol. Samples were assayed by reversed-phase high-performance liquid chromatography with electrochemical detection. Dihydroxybenzylamine was used as the internal standard. The mobile phase was a citrate/acetate buffer containing 5% (vol/vol) MeOH, pH 4.4. The citrate/acetate buffer was of the following composition (mmol/L): sodium acetate trihydrate 50.0, citric acid monohydrate 20.0, sodium octane sulfonic acid 3.75, sodium EDTA 0.135, and di-n-butylamine 1.0. Flow rate was 1 mL/min, and 50-μL aliquots of sample were injected onto the column (C18 5 μm Resolve, Waters Chromatography Division).

**Data Analysis**

Phenylephrine-mediated constriction was quantified by determining the area under the phenylephrine-constriction curve using a trapezoidal method of analysis. Relaxation in response to isoproterenol or nitroprusside was quantified by determining the area above the relaxation response curve using a trapezoidal method of analysis (Fig 1).

The relaxation response at a single time point was expressed as a percentage of maximal phenylephrine-generated tension. The effects of insulin on both contraction and relaxation responses were expressed as a percentage of baseline response normalized for temporal changes as assessed in the parallel control ring segments. Results are expressed as mean±SEM. Statistical analysis was by one-way ANOVA and Student's or Welch's t test for unpaired data. A value of P<.05 on a two-tailed test was taken as the minimum level of significance.

**Results**

The addition of phenylephrine to isolated ring segments from Wistar rats resulted in an initial rapid development of tension and sustained steady plateau (Fig 1). The addition of isoproterenol to maximally phenylephrine-preconstricted rings resulted in a rapid relaxation, reaching a nadir by 4 minutes followed by a gradual, partial recovery of tension that remained stable for more than 30 minutes (Fig 1).

We considered whether the partial recovery of tension after isoproterenol addition might be due to degradation of the drug. However, a second addition of isoproterenol 20 minutes after the initial addition did not significantly augment the relaxation response (42.7±3.6% of maximal tension before second addition of isoproterenol to 36.1±2.9% of maximal tension after second addition, n=4; P=.15 for paired observations). Additionally, for direct assessment of the stability of isoproterenol degradation, samples were taken after drug addition, and bath concentrations were assessed by high-performance liquid chromatography. No significant reductions in organ bath isoproterenol concentration were evident throughout the duration of the experiment (Fig 2).

To determine whether insulin altered α-adrenergic vasoconstriction in isolated ring segments, phenylephrine-mediated constriction was examined. Insulin (0.01 to 10.0 nmol/L) exposure did not affect phenylephrine-mediated constriction (Fig 3).

To assess whether insulin altered β-adrenergic-mediated vasorelaxation, we examined the effects of isopro-
terenol. Insulin (0.01 to 10.0 nmol/L) was associated with a concentration-dependent increase in the extent of isoproterenol-mediated vasorelaxation in phenylephrine-preconstricted rings (Figs 4 and 5). The EC_{50} for the insulin effect was 32 pmol/L, with a maximum of 20-45% above baseline isoproterenol-mediated relaxation (n=12, P<.001). As noted above, isoproterenol-mediated relaxation followed a biphasic response, with an initial maximal phase of relaxation (up to 4 minutes after addition) followed by a partial recovery of tension that then remained stable throughout the test period. To further assess the role of insulin on β-adrenergic responses, we considered isoproterenol-mediated relaxation during the “onset” phase (0 to 4 minutes) and “recovery” phase (4 to 20 minutes). As shown in Table 1, insulin-mediated augmentation of isoproterenol-mediated response was accounted for (primarily) by enhancement of relaxation during the recovery phase of the response (to a maximum of 29±7%, with an EC_{50} of 25 pmol/L).

We next examined the role of the endothelium in both isoproterenol-mediated relaxation and its enhancement by insulin. After endothelium removal, maximal isoproterenol-mediated relaxation (in the absence of insulin) was unaltered (nadir effect, endothelium-denuded rings: 30.7±7.7% of maximal tension, n=6; endothelium-intact rings: 28.2±4.1% of maximal tension, n=17; P=.77). However, the extent of the “rebound” recovery in tension was exaggerated in endothelium-denuded rings (effect at 20 minutes, endothelium-denuded rings: 77.8±4.8% of maximal tension; endothelium-intact rings: 60.5±4.5% of maximal tension; P=.04). In addition, overall isoproterenol-mediated relaxation during the recovery phase, as derived from area above the curve calculations from 4 to 20 minutes, was decreased in endothelium-denuded ring segments (Table 2). Furthermore, in endothelium-denuded rings, human insulin (0.1 to 10.0 nmol/L) did not significantly enhance isoproterenol-mediated relaxation (Fig 6).

To assess whether insulin-mediated enhancement of β-adrenergic-mediated relaxation reflected a generalized enhancement in vasorelaxation, we also examined sodium nitroprusside-mediated relaxation. The addition of sodium nitroprusside to maximally phenylephrine-preconstricted ring segments (in the absence of insulin) resulted in a rapid relaxation, reaching a nadir by 4 minutes (to 15.0±4.2% of maximal tension, n=6) followed by a gradual, partial recovery of tension (effect at 20 minutes after addition, 27.6±10.7% of maximal tension). Insulin (0.01 to 10.0 nmol/L) did not significantly affect sodium nitroprusside-mediated relaxation in phenylephrine-precontracted rings (Fig 7).

The effect of human insulin on isoproterenol-mediated relaxation was examined in rings obtained from age-matched SHR maintained under the same conditions as the Wistar rats. At the time of study, systolic blood pressures (determined with tail-cuff techniques) were significantly higher in SHR compared with those measured in concurrently studied Wistar rats (142±3 versus 90±2 mm Hg, SHR [n=10] versus Wistar [n=5]; P<.001). The extent of phenylephrine-mediated tension developed was marginally increased in Wistar rats compared with those obtained in SHR (550±35 versus 469±29 mg, Wistar [n=30 rings] versus SHR [n=20 rings]; P=.08 by Welch’s t test for two populations with unequal variances).

In phenylephrine-precontracted SHR aortic ring segments, addition of isoproterenol resulted in an initial relaxation (reaching a nadir by 4 minutes of 48.8±4.1% of maximal tension, n=10) followed by a partial recovery of tension (effect at 20 minutes after addition, 73.9±1.9% of maximal tension). Although the pattern of relaxation and rebound in SHR aortic ring segments was qualitatively similar to that observed in Wistar rat segments, the extent of both nadir relaxation and relaxation after partial recovery was significantly attenuated in SHR compared with Wistar rats (nadir, P=.0001, and recovery, P=.009 by Welch’s t test versus the effects of isoproterenol on Wistar rings). As in ring segments from Wistar rats, insulin did not affect the extent of phenyle-

**Table 1. Effect of Insulin on Onset and Recovery Phases of Isoproterenol in Wistar Thoracic Aortic Ring Segments**

<table>
<thead>
<tr>
<th>Insulin, nmol/L</th>
<th>Onset, %</th>
<th>Recovery, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>102±2</td>
<td>107±7</td>
<td>5</td>
</tr>
<tr>
<td>0.1</td>
<td>108±2</td>
<td>122±6*</td>
<td>15</td>
</tr>
<tr>
<td>1.0</td>
<td>104±3</td>
<td>122±5*</td>
<td>14</td>
</tr>
<tr>
<td>10.0</td>
<td>109±3*</td>
<td>129±7*</td>
<td>12</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of baseline isoproterenol-mediated relaxation and represent mean±SEM. n indicates number of experiments.

*P<.05 vs baseline isoproterenol-mediated relaxation by one-way ANOVA.
β-adrenergic-mediated relaxation in aortic ring segments obtained from SHR (Fig 8 and Table 3).

Discussion
In the present study we demonstrated that acute exposure to insulin selectively enhances β-adrenergic responsiveness in aortic ring segments from Wistar rats but not in aortas obtained from SHR. Furthermore, the observed response to insulin was dependent on the presence of a functionally intact endothelium.

Isoproterenol-mediated relaxation in phenylephrine-preconstricted rings followed two distinct phases. A peak relaxation phase was followed by a phase characterized by partial recovery of tension that then remained stable throughout the observation period. A biphasic response to isoproterenol, similar to that observed in our studies, has been previously reported in ring segments from rabbit aortas. The authors of that study concluded that the recovery of tension was due to desensitization of the β-adrenergic receptor and not to the degradation of isoproterenol. Consistent with this conclusion, our results indicate that the loss of relaxant response was not due to isoproterenol degradation. Furthermore, the lack of any augmentation of the relaxant response after a second addition of isoproterenol is consistent with the hypothesis that the recovery in tension is due to desensitization. Additionally, the extent of attenuation and the time course for this process parallel the time course for in vitro desensitization of

β-adrenergic-mediated adenyl cyclase activity observed in cell model systems. Notably, a rebound in tension was also noted after nitroprusside-mediated relaxation (although to a lesser extent than that observed after isoproterenol administration). Previous investigators have ascribed this effect to desensitization of guanylyl cyclase activation.

Insulin exposure selectively enhances isoproterenol-mediated relaxation without altering phenylephrine-mediated constriction or nitroprusside-mediated relaxation. Previous studies have reported variable effects of insulin on α-adrenergic-mediated vasocostriction. High insulin levels have been associated with increased responsiveness in rat mesenteric vasculature and decreased responsiveness in rat mesenteric arterioles and rabbit femoral arteries and veins. The explanation for these differences is unclear but could reflect differences in species, vascular beds, type of insulin, or duration of insulin exposure. It is notable that in our study the effect of insulin was assessed without a period of pretreatment, whereas previous studies used longer durations of preexposure (30 to 90 minutes).

The effect of insulin was most pronounced in the later phase of isoproterenol-mediated relaxation, the stage characterized by a partial recovery of tension. As discussed above, previous authors, observing a similar phenomenon in rabbit aortic ring segments, ascribed it to β-adrenergic desensitization. Our findings would support this hypothesis. If the partial recovery of tension was prolonged isoproterenol exposure does represent β-adrenergic desensitization, the effect of insulin in enhancing β-adrenergic response could be via attenua-

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**Table 2. Comparison of Area Above the Curve During Overall, Onset, and Recovery in Endothelium-Intact and Endothelium-Denuded Wistar Thoracic Aortic Ring Segments**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Overall</th>
<th>Onset</th>
<th>Recovery</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelium-intact</td>
<td>1012±78</td>
<td>243±12</td>
<td>793±87</td>
<td>17</td>
</tr>
<tr>
<td>Endothelium-denuded</td>
<td>785±109</td>
<td>238±24</td>
<td>547±89</td>
<td>6</td>
</tr>
</tbody>
</table>

Area above the curve is expressed as percent tension x minute and represents mean±SEM. n indicates number of experiments. Statistical analysis was by one-way ANOVA. P value is for comparison of responses in endothelium-intact vs endothelium-denuded groups.

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**Figure 6.** Bar graph shows effect of varying insulin concentrations on isoproterenol-mediated relaxation in endothelium-denuded ring segments. Values are expressed as percentage change from baseline isoproterenol-mediated relaxation and represent mean±SEM; n=6 at each insulin concentration. ND indicates not determined.

**Figure 7.** Bar graph shows effect of varying insulin concentrations on sodium nitroprusside-mediated relaxation. Values are expressed as percentage change from baseline nitroprusside-mediated relaxation and represent mean±SEM; n=6 at each insulin concentration.

**Figure 8.** Bar graph shows effect of 10 nmol/L insulin on isoproterenol-mediated relaxation in ring segment from spontaneously hypertensive rats (SHR) compared with effect of insulin on ring segments from Wistar rats. Values are expressed as percentage change from baseline isoproterenol-mediated relaxation and represent mean±SEM; n=10 for SHR and n=12 for Wistar rats. *P<.05 comparing change in isoproterenol-mediated relaxation in SHR vs Wistar rats.
Ontario. Career Investigator of the Heart and Stroke Foundation of Ontario. Dr. Feldman is a contributor to the defect in β-adrenergic-mediated vasoresistance, then an impairment in this mechanism could contribute to the defect in β-adrenergic-mediated vasodilation in SHR. 

Whether the lack of any detectable enhancement of β-adrenergic responsiveness by insulin in SHR is due to impaired β-adrenergic-mediated effects, impaired endothelium-mediated relaxation, impaired insulin-mediated responses, or a combination of the three remains to be determined. However, regardless of the mechanism, if insulin-mediated sensitization of β-adrenergic-mediated vasodilation occurs in vivo and is important in the maintenance of normal peripheral resistance, then an impairment in this mechanism could contribute to the defect in β-adrenergic-mediated vasodilation that occurs in both human and animal models of hypertension.

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References

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