Experimentally Induced Acute Hyperinsulinemia Stimulates Endogenous Nitric Oxide Production in Humans: Detection Using Urinary NO_2^-/NO_3^- Excretion

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Insulin-mediated glucose metabolism in skeletal muscle is associated with a proportional increase in muscle perfusion. The vasodilatory effect of insulin is thought to be mediated in part by endothelium-derived nitric oxide (NO). The present study was performed to determine whether acute hyperinsulinemia has any stimulatory effect on endogenous NO production in humans. Bolus intravenous injection of insulin (0.1 IU/kg body weight) caused a significant increase in urinary excretion of NO_2^-/NO_3^- together with a significant decrease in blood pressure, whereas saline infusion alone had no effect on these parameters. The increased NO response to insulin was almost comparable to that obtained with infusion of 30 g L-arginine. The acute effect of hyperinsulinemia on endogenous NO formation supports the concept that NO may mediate the vasodilatory action of insulin in humans.

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IN ADDITION TO its action on tissue glucose metabolism, insulin also influences the cardiovascular system and may play a role in the regulation of blood pressure.[1,2] Accumulating evidence suggests that insulin has a direct effect on blood vessels and induces vasodilation and capillary recruitment of skeletal muscles. These changes increase blood flow and insulin and glucose delivery to the muscle, thus contributing substantially to insulin-induced glucose uptake.[1,2] Insulin appears to have a direct relaxing effect on vascular smooth muscle through several local mechanisms.[1-5] These include (1) stimulation of β-adrenergic receptors, (2) stimulation of the Na^+/K^+ pump with hyperpolarization of vascular smooth muscles, (3) increased Ca^{2+}–adenosine triphosphatase activity, and/or (4) metabolic vasodilation secondary to increased muscle oxygen consumption. In addition, several recent reports have suggested that endothelium-derived nitric oxide (NO) may also play a role in mediating the effect of insulin on vascular smooth muscle tone.[2,6,7]

NO plays a pivotal role in the regulation of vascular tone and regional blood flow.[8,9] NO synthesized from L-arginine by NO synthase in the vascular endothelium diffuses into the underlying smooth muscles and activates soluble guanylate cyclase to generate cyclic guanosine monophosphate, which in turn causes relaxation of smooth muscles and vasodilation. Since NO is a labile substance, direct measurement of NO is difficult, particularly in vivo.[8,9] NO decomposes rapidly in biological solutions into more stable NO_2^- and NO_3^- (NO_x^-) that can be measured as markers of endogenous production of NO.[10-12]

The effects of acute hyperinsulinemia on whole-body endogenous NO formation have not been investigated. In the present study, we examined the acute effect of intravenously administered insulin on endogenous NO production in humans.

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SUBJECTS AND METHODS

Subjects

Eight healthy non-obese but short children (four boys and four girls, aged 3.2 to 13.9 years) participated in this study after provision of informed consent. The tests were performed to evaluate growth hormone secretion in each subject. All the children tested were finally diagnosed as having nonendocrine short stature. We also studied another group of four normal non-obese children (two boys and two girls aged 6.0 to 11.0 years (mean, 8.5) who served as normal control subjects. All subjects were normotensive, were not taking any medication, and had no evidence of metabolic or cardiovascular disease and no family history of diabetes. Routine analyses of blood chemistry and hematology were within normal limits.

The studies began at 8:00 AM in a quiet room with a constant temperature of 22° to 24°C. The subjects, who fasted overnight for at least 12 hours, passed urine and lay supine. A 24-gauge polyethylene catheter was inserted into the dorsal vein of the hand for infusion of physiological saline (0.9% NaCl) at a constant rate of 0.8 mL/min. Measurement of arterial blood pressure was performed every 15 minutes by an automatic self-inflating sphygmomanometer (Nippon Korin, Tokyo, Japan) throughout each study protocol.

Protocol 1

After a 60-minute rest period, five test children (two boys and three girls aged 3.2 to 13.9 years [mean, 9.5]) passed the first urine sample at 9:00 AM (for baseline measurement), and then a bolus dose of 0.1 IU/kg body weight regular insulin (Novolin R; Novo-Yamanouchi, Tokyo, Japan) was injected intravenously. Physiological saline was subsequently infused at a constant rate of 0.8 mL/min over 120 minutes. The study was terminated at the end of saline infusion, and the subject was asked to provide another sample of urine.

In our subjects, six venous blood samples were taken via the catheter for plasma glucose determination (by the glucose oxidase method) at baseline and then 15, 30, 60, 90, and 120 minutes postinjection. Injection of Novolin R caused a rapid decrease in plasma glucose followed by a return toward basal, with the hypoglycemic nadir in each case occurring at 15 or 30 minutes ($P < .005$ and $P < .001$ *v* baseline, respectively, paired *t* test). Plasma glucose concentrations were as follows: 75.4 ± 6.2 mg/dL (baseline), 41.0 ± 7.3 mg/dL (15 minutes), 50.2 ± 5.3 mg/dL (30 minutes), 68.4 ± 9.1 mg/dL (60 minutes), 74.8 ± 5.6 mg/dL (90 minutes), and 77.6 ± 8.3 mg/dL (120 minutes). According to the manufacturer's data, bolus injection of Novolin R (0.1 IU/kg) results in an immediate increase from basal of serum insulin concentrations, reaching peaks of approximately 1,000 μU/mL, followed by a return toward basal by 60 minutes postinjection.

The test was also performed in four control subjects, but insulin was not injected. Instead, physiological saline was infused at a rate of 0.8

mL/min throughout the 3-hour test period extending from 8:00 to 11:00 AM. Urine samples were collected in a similar fashion.

Protocol 2

The test was performed in five test children (two boys and three girls aged 10.3 to 13.9 years (mean, 12.1) and included two girls who underwent the insulin study of protocol 1 on another day. Following a rest period of 60 minutes, the children passed the first urine sample, and 30 g L-arginine HCl dissolved in 300 mL distilled water (Morishita, Osaka, Japan) was then infused over 30 minutes. Physiological saline (0.8 mL/min) was subsequently infused over 90 minutes commencing at 9:30 AM. At the end of saline infusion, each subject was asked to provide a second sample of urine.

Five venous blood samples were taken via the catheter for serum insulin determination (by radioimmunoassay) during the basal period and thereafter every 30 minutes up to 120 minutes after the start of L-arginine administration.

All urine samples were centrifuged immediately, and the supernatant was stored at −80°C until analysis. The first and second urine samples were analyzed for urinary NO_x^- concentration in all subjects.

Measurement of Urinary NO_x^- Levels

We measured the stable metabolic products of NO (ie, NO_x^-) in the urine in duplicate using the brucine method.[13,14] Briefly, we prepared a 2:1 dilution of NO_3^--free concentrated H_2SO_4 (Wako, Osaka, Japan) to which 60 mg/100 mL strychnine (Sigma Chemicals, St Louis, MO) was added. Urinary NO_2^- was oxidized to NO_3^- by titration with 0.3% $KMnO_4$ until a slightly purple color was evident. After 30 minutes, titration with 1.0 mol/L $NaHSO_3$ was performed to decolorize the sample. Four milliliters of the strychnine/H_2SO_4 solution was then added to 1.0 ml of the unknown or standard solution. The mixture was heated to boiling for 10 minutes and then transferred to an ice bath for 20 minutes. The reaction yielded a sulfur color, which was read on a spectrophotometer (Titertek Multiskan MCC; Flow Laboratories, Costa Mesa, CA) at 405 nm. Standard curves were established with serial dilutions of $NaNO_3$. The detection limit of NO_x^- was 10 µmol/L, and the overall assay variance was less than 5%. The level of NO_x^- was expressed relative to urinary creatinine concentration using the Jaffé method.

The level of NO_x^- was also determined in the early-morning urine samples of the test and control children, representing spontaneous overnight production of NO. The concentration of NO_x^- in these samples ranged from 0.110 to 0.309 mmol/mmol Cr (mean, 0.188). These values were considered within the normal range based on data reported recently by our laboratory.[15]

Statistical Analysis

The data are expressed as the range and mean or mean ± SD. Statistical analysis was performed using the paired or unpaired *t* test where appropriate. A *P* value .05 denoted statistical significance.

RESULTS

Blood Pressure

Administration of insulin (protocol 1) resulted in a significant decrease in mean arterial blood pressure from a baseline level of 82 ± 4 mm Hg to 75 ± 6 mm Hg at 15 minutes ($P < .005$, paired *t* test). However, blood pressure gradually returned to the baseline level in all test subjects (Fig 1). Saline infusion alone induced no significant changes in mean arterial blood pressure in four control subjects.

The mean blood pressure decreased significantly during infusion of L-arginine (protocol 2) from 68 ± 12 mm Hg at baseline to 62 ± 12 mm Hg at 30 minutes ($P < .005$, paired *t* test) in all test subjects. A prompt return to the baseline level occurred after the end of L-arginine infusion (Fig 1).

Urinary NO_x^- Excretion

The effects of administration of insulin, L-arginine, and saline alone on urinary NO_x^- excretion are shown in Fig 2. Urinary NO_x^- excretion increased significantly after administration of insulin and L-arginine ($P < .005$ and $P < .05$, respectively, paired *t* test), whereas only a small insignificant increase in NO_x^- was observed after saline alone.

We also calculated the percent increase in urinary NO_x^- concentration. For this purpose, the posttreatment concentration

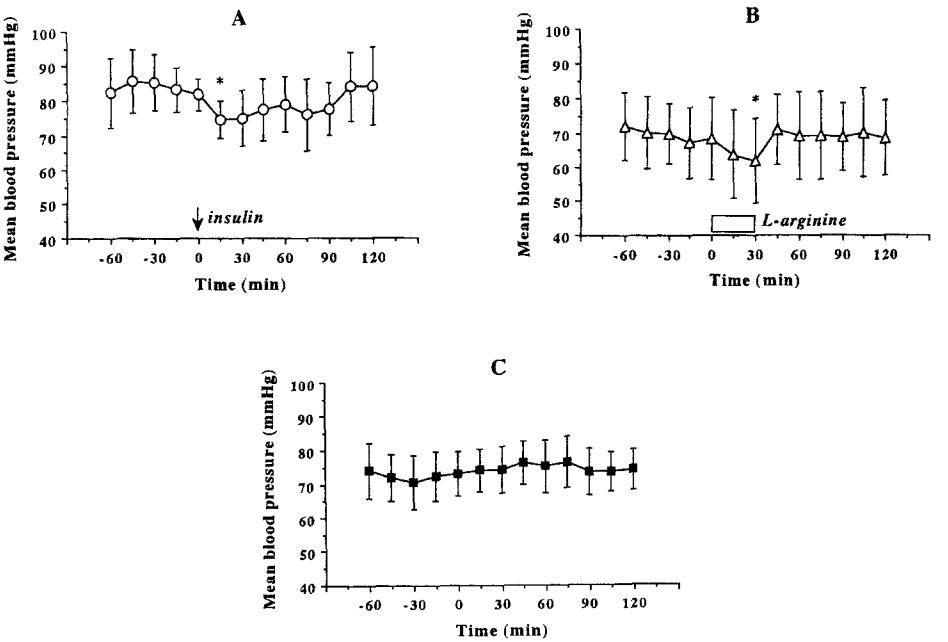

Fig 1. Effects of administration of insulin (A), L-arginine (B), and saline (C) on mean arterial blood pressure. Data are the mean ± SD. *$P < .005$ *v* baseline (paired *t* test).

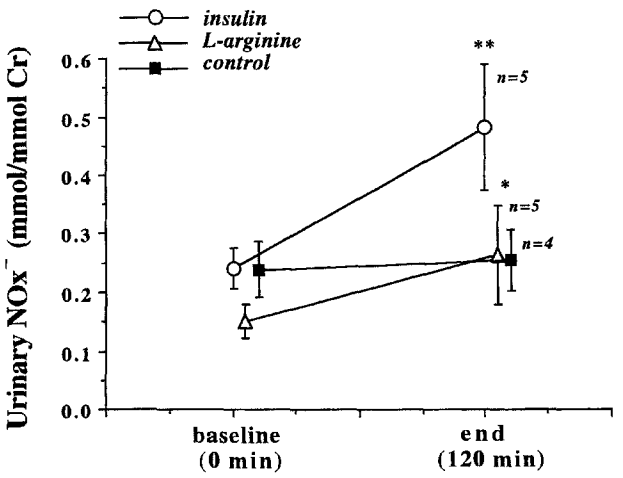

Fig 2. Changes in urinary NO_x^- levels produced by (○) insulin, (△) L-arginine, and (■) saline administration. Data are the mean ± SD. *$P < .05$ and **$P < .005$ *v* baseline (paired *t* test).

was expressed as a percent of the baseline value for each treatment. There was a 101%, 71%, and 7% increase in urinary NO_x^- at the end of the observation period relative to baseline following administration of insulin, L-arginine, and saline alone, respectively (Fig 3).

Alterations in Serum Insulin Concentrations in Protocol 2

Administration of L-arginine (protocol 2) resulted in a significant increase in serum insulin from a baseline level of 5.0 ± 1.6 μU/mL to 55.7 ± 40.1 μU/mL at 30 minutes ($P < .05$ *v* baseline, paired *t* test) followed by a return toward basal (Fig 4).

DISCUSSION

The hemodynamic effects of insulin have recently received a great deal of attention.[1,2] Recent studies have focused on the role of insulin as an endogenous vasodilator. The combined use of the hyperinsulinemic-euglycemic clamp and the limb-balance technique (to measure limb blood flow and the arterio-venous glucose gradient) has shown that hyperinsulinemia in

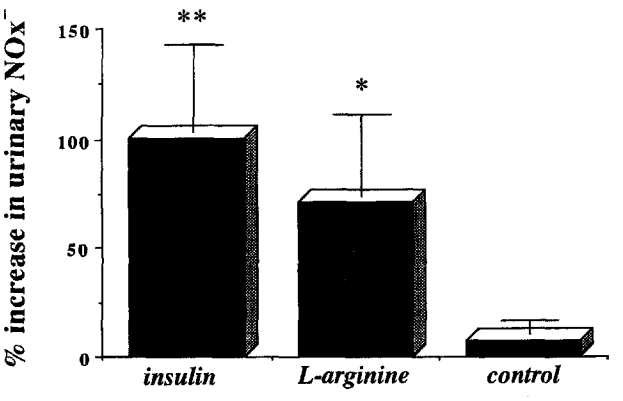

Fig 3. Percent increase in urinary NO_x^- levels produced by insulin, L-arginine, and saline administration. Data are the mean ± SD. *$P <$ .05 and **$P < .005$ *v* saline alone (unpaired *t* test).

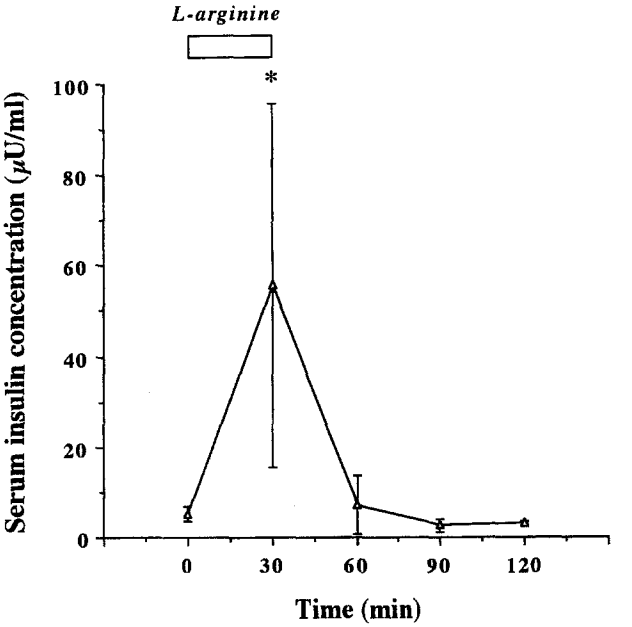

Fig 4. Changes in serum insulin concentrations produced by L-arginine administration. Data are the mean ± SD. *$P < .05$ *v* baseline (paired *t* test).

lean humans can double the blood flow to the leg and that the blood insulin concentration required to produce a half-maximal vasodilatory response is well within the physiological range.[2,16,17] Moreover, insulin appears to cause a preferential dilatation of arterioles that supply skeletal muscles via an endothelium-derived NO-dependent mechanism. This is supported by the finding that *N*-monomethyl-L-arginine, a specific inhibitor of NO synthase, causes a larger reduction in limb blood flow during hyperinsulinemia than at baseline.[6,7] This hemodynamic response seems to be impaired in insulin-resistant states such as obesity, type II diabetes, and hypertension.[16-18]

The abundance of insulin receptors in small arteriolar and capillary endothelium suggests the presence of specialized features of endothelium that are likely to contribute to the unique interactions of endothelial cells with insulin.[2,19] Very recently, Zeng and Quon[20] have provided direct evidence for insulin-stimulated NO production from human umbilical vein endothelial cells, using an amperometric NO-selective electrode. The effects of insulin on NO production appear to be mediated in part through the insulin receptor using a wortmannin (an inhibitor of phosphatidylinositol 3-kinase)-dependent pathway.

However, until now, the levels of NO or its stable metabolite (ie, NO_x^-) have not been determined in hyperinsulinemia in humans. The main purpose of the present study was to examine whether acute hyperinsulinemia caused by intravenously administered exogenous insulin has any stimulatory effect on endogenous NO production. Bolus injection of insulin (0.1 IU/kg body weight) significantly increased urinary NO_x^- excretion in five children; the posttreatment concentration was approximately double that recorded at baseline. Insulin administration caused a simultaneous and significant, albeit transient, decrease in mean arterial blood pressure (mean, −7 mm Hg). On the

other hand, maintenance infusion (0.8 mL/min saline) alone had no significant effects on urinary NO_x^- excretion and blood pressure, as shown in the other four control children. Considered together, our results suggest that acute hyperinsulinemia can stimulate endogenous NO production in humans under resting conditions. Our finding provides support for the results of Steinberg et al[6] and Scherrer et al[7] suggesting that NO plays a critical role in mediating the vasodilatory action of insulin. Such a vasodilatory effect may offset the sympathetic and antinatriuretic (ie, pressor) actions of this hormone in regulating arterial blood pressure.[1,2]

As described elsewhere,[10,12,15] urinary NO_x^- levels reflect not only NO produced by constitutive enzymes involved in basal vasoregulation and neurotransmission, but also NO formed by the activated inducible NO synthases. In this regard, our methods could not define the exact sites or isoforms of NO synthase measured in the urinary NO_x^- excretion test. Urinary NO_x^- could also derive from the urogenital tract.[21] However, the swiftness with which insulin administration increased urinary NO_x^- excretion in our subjects in the absence of exogenous NO_x^- sources (eg, diet) and the abundance of insulin receptors in vascular endothelial cells[2,19] suggest that acute hyperinsulinemia activates the constitutive NO synthase in the vascular endothelium.

The stimulatory effects of L-arginine, a substrate of NO synthesis, on the NO system have also been shown in humans by others.[10,22] The present study confirmed these early data by demonstrating a significant decrease in mean blood pressure with a concomitant increase in urinary NO_x^- excretion. We also found that the NO response to insulin administration was almost comparable to the response to a large dose of L-arginine, although the NO response to each stimulus could have been influenced by the age difference observed between the two groups. L-Arginine is also known to stimulate insulin release by the pancreas.[23] Circulating insulin levels were increased in our subjects during infusion of L-arginine. Thus, the enhanced NO production observed in the L-arginine test might represent the combined effect of NO synthase substrate provision and the hyperinsulinemic state.

In conclusion, our study demonstrates for the first time that experimentally induced acute hyperinsulinemia stimulates whole-body endogenous NO formation in humans. Our results support the concept that the vasodilatory action of insulin is mediated at least in part by the NO pathway, and that states of insulin resistance may be associated with a defect in insulin's action to modulate the NO system.[2]

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