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Original Research Article

Nitric Oxide: The "Second Messenger" of Insulin

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Summary

Incubation of various tissues, including heart, liver, kidney, muscle, and intestine from mice and erythrocytes or their membrane fractions from humans, with physiologic concentration of insulin resulted in the activation of a membrane-bound nitric oxide synthase (NOS). Activation of NOS and synthesis of NO were stimulated by the binding of insulin to specific receptors on the cell surface. A Lineweaver–Burk plot of the enzymatic activity demonstrated that the stimulation of NOS by insulin was related to the decrease in the K_m for L-arginine, the substrate for NOS, with a simultaneous increase of V_{max}. Addition of N^G-nitro-L-arginine methyl ester (LNAME), a competitive inhibitor of NOS, to the reaction mixture completely inhibited the hormone-stimulated NO synthesis in all tissues. Furthermore, NO had an insulin-like effect in stimulating glucose transport and glucose oxidation in muscle, a major site for insulin action. Addition of NAME to the reaction mixture completely blocked the stimulatory effect of insulin by inhibiting both NO production and glucose metabolism, without affecting the hormone-stimulated tyrosine or phosphatidylinositol 3-kinases of the membrane preparation. Injection of NO in alloxan-induced diabetic mice mimicked the effect of insulin in the control of hyperglycemia (i.e., lowered the glucose content in plasma). However, injection of NAME before the administration of insulin to diabetic-induced and nondiabetic mice inhibited not only the insulin-stimulated increase of NO in plasma but also the glucose-lowering effect of insulin.

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Keywords L-NAME; nitric oxide synthase; phosphatidylinositol 3-kinase; red blood cells; tyrosire kinase.

INTRODUCTION

Insulin has an essential role in carbohydrate metabolism. Its effects, however, are not restricted to the stimulation of carbohydrate metabolism in insulin-responsive cells. The hormone is known for its influence on various biochemical and pathological events, including carbohydrate, fat, and protein metabolism (*1*, *2*); atherosclerosis (*3*); thrombosis (*4*); and neuropathological events (*5*). As with other hormones, the effects of insulin are the consequence of the binding of the protein molecules to the target tissue (*6*). However, the molecular events that follow insulin interaction and result in the transduction of insulin effect into the cell remain obscure. What has been established is that the interaction of insulin with specific cell surface receptors results in the activation of insulin receptor tyrosine kinase (*7*), which is thought to modify the biologic activity of various proteins through downstream phosphorylation by the activated enzyme. Unfortunately, however, the identity of neither the protein substrate for the enzyme nor any product(s) that may mediate the transmembrane signaling of the insulin effect as its messenger has yet been established. Similarly, the activation of membrane phosphatidylinositol 3-kinase (PI 3-kinase)[4] by insulin has been demonstrated to be involved in the hormone signaling pathway (*8*). However, the identity of the "messenger" so far has not been identified. Whether the insulin receptor interaction would actually lead to the synthesis of any messenger capable of eliciting insulin-mimetic properties in the hormone-responsive cells remains uncertain. Compounds such as vanadate (*9*) or H_2O_2 (*10*) have been shown to exhibit insulin mimetic activity. The in vivo formation of vanadium compounds as an insulin-activated reaction does not occur, and the formation of H_2O_2 by insulin remains controversial (*11*).

Recently, we showed that administration of physiologic concentrations of insulin in mice resulted in the reduction of the blood glucose content with a simultaneous increase in plasma methemoglobin (*12*). Because the formation of methemoglobin after the insulin injection suggested the formation of nitric oxide

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[4]Abbreviations: RBC, red blood cells; NAME, N^G-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PI 3-kinase, phosphatidylinositol 3-kinase.

(NO) in the system (*13*), we tested the possibility as to whether the increase of NO in the blood was cause by activation of nitric oxide synthase (NOS) by the hormone and, if so, whether the NO thus formed could have any "messenger" role for the insulin effect. In this report we demonstrate that insulin at physiologic concentrations specifically activates a membrane-bound constitutive NOS. The NO formation catalyzed by the NOS produced insulin-like effects on carbohydrate metabolism both in vitro and in vivo.

MATERIALS AND METHODS

Ethical Clearance

The protocol was approved by the institutional review board, Veterans Affairs Medical Center, Bronx, NY, and Jadavpur University.

Materials

N^G-Nitro-L-arginine methyl ester (NAME), sodium nitroprusside, alloxan, protein A, antibodies to PI 3-kinase and phosphotyrosine, Glu^{80}-Tyr^{40} copolymer, inositol monophosphate, 2-deoxy-D-^{14}C-glucose (210 mCi/mmol), and [U-^{14}C]D-glucose (310 mCi/mmol) were obtained from Sigma Chemical Co. Anti-insulin receptor antibody was prepared from the serum of a Type II diabetic patient and was a gift from Dr. T. K. Roy, Temple University, Philadelphia, PA. [γ-^{32}P]ATP, specific activity 10 mCi/mmol, was a product of Amersham.

Animal Groups

Inbred albino mice (Swiss Webster) of both sexes were raised in the animal facility of our laboratory (Jadavpur Univ.) from birth to age 2 months. The mice were housed with free access to feed and water under 12-h cycles of alternating light and dark. A total of 40 mice were studied. The mice, weighing 18–23 g, were divided into six groups. Group 1 consisted of 10 mice from which various tissue samples were taken; these samples were prepared as homogenates, incubated with insulin (HumulinR; Eli Lilly, Indianapolis, IN), and used to detect NO formation as well as in other studies. The remaining 30 mice were made diabetic with alloxan and divided into five groups of six animals each. Group 2 received insulin (200 μU/ml); Group 3, NO (600 pmol/g body wt); Group 4, saline only (controls); Group 5, NAME (10 μM in saline), followed by treatment with insulin; Group 6, NAME (10 μM), followed by NO treatment.

Human Samples

Blood was collected from six healthy nondiabetic, normotensive volunteers (three men and three women) with no known family history of diabetes or hypertension. None of the volunteers had taken any medication for at least 2 weeks before blood donation. Blood was collected in plastic tubes by mixing 9 volumes of blood with 1 volume of 0.13 M sodium citrate. Intact red blood cells (RBC) were suspended in 0.9% NaCl, and RBC membranes were prepared by ultracentrifugation of RBC lysate (*14*).

Methods

Preparation of Tissue Homogenate and Membrane Fractions from Mice. Nondiabetic adult albino Swiss mice were killed by cervical dislocation. Various tissues, including heart, liver, and kidney, were homogenized in an all-glass Potter–Elvehjem homogenizer by suspending different tissues (5 mg/ml) in Krebs buffer, pH 7.4, at 0 °C. The homogenized mass was then centrifuged at 60 000 × *g* at 0 °C for 60 min. The supernatant and the particulate fractions containing membranes were collected separately. Before use, the particulate fraction was suspended in equal volume of the same buffer (*15*). Unless otherwise stated, all incubations were carried out in Krebs buffer, pH 7.4, containing 2.0 mM $CaCl_2$.

Incubation of Different Intact Tissues. Intact tissues from rat intestine, heart, muscle, liver, and kidney, and human RBCs were incubated in a total volume of 2.5 ml in Krebs buffer, pH 7.4, containing 10 μM L-arginine, 2.0 mM Ca^{2+}, and various concentrations of insulin. The samples were incubated at 37 °C for 30–45 min with occasional stirring. After incubation the suspension was collected and clarified by centrifugation at 8000 × *g* for 10 min at 0 °C; the NO content of the supernatant was then determined as described below.

Assay of NOS Activity in Tissue Homogenates and Membrane Preparations. The reaction mixture containing either soluble supernatant or particulate fractions (2 to 3 mg of protein) was incubated in a total volume of 2.5 ml of Krebs buffer, pH 7.4, containing 2.0 mM Ca^{2+}, 10 μM L-arginine, and various concentrations of insulin for different periods at 37 °C, with constant stirring. At timed intervals, portions of the reaction mixture were centrifuged at 8000 × *g* for 5 min at 0 °C, and the NO content of the supernatant was determined.

Determination of NO Formation. The formation of NO was quantitated by determining the conversion of methemoglobin as described by Jia et al. (*13*). Typically, 1.0-ml supernatant fractions obtained from the incubation mixture were added to a preparation of oxyhemoglobin (64 mM, final concentration) under N_2 (*15*). Spectral changes for the conversion of oxyhemoglobin to methemoglobin was monitored by using a scanning Beckman spectrophotometer (Model DU6). From the decrease of absorbance at 575 nm and 650 nm maxima, NO formation was determined. Amounts of NO in the reaction mixture were confirmed independently by a chemiluminescence technique (*16*).

Binding of Insulin to Purified RBC Membrane and the Synthesis of NO. Human RBC membranes were incubated with 2.0 nM ^{125}I-labeled insulin for 60 min at 23 °C, as described (*14*). The specific binding of insulin to the RBC membrane was determined by adding excess unlabeled insulin (0.2 μM) to the suspension and subtracting nonspecific binding from the total binding, making appropriate corrections for the degradation of insulin (*14*). The binding of insulin to the membrane preparation was determined by adding different amounts of unlabeled insulin

to a constant amount of ^{125}I-labeled insulin; the specific binding was calculated from the total concentration of the hormone added to the binding mixture. In parallel experiments, synthesis of NO by the RBC membrane stimulated with unlabeled insulin was determined. Dissociation constants (K_{d_1} and K_{d_2}) and the receptor numbers (n_1 and n_2) for insulin binding to its receptors on the RBC membrane were determined by Scatchard plot (*17*). The RBC suspension was also incubated with purified anti-insulin receptor antibody (78 μg/10^8 cells) in Krebs buffer for 90 min at 37 °C to block the insulin receptor binding sites on the cell surface. After incubation, RBC membranes were prepared and the binding of insulin to the membrane and the synthesis of NO in the presence of insulin were determined.

Determination of Glucose Transport. Glucose transport activity was determined by using epitrochlearis muscle from mice as a model for target tissue for insulin action (*18*). The glucose transport activity was determined by using 2-deoxy-D-^{14}C-glucose. Typically, epitrochlearis muscle (0.4–0.7 g) from non-diabetic albino mice was incubated at 37 °C with 10 nmol of 2-deoxy-D-^{14}C-glucose (310 μCi/mmol) in 1.0 ml of Krebs–Ringer buffer, pH 7.4, containing 10 mg/ml bovine serum albumin plus different concentrations of either insulin or NO in 0.9% NaCl in the presence or absence of 10 μM NAME. The glucose transport activity was determined as described elsewhere (*18*).

Determination of Glucose Oxidation. Mice epitrochlearis muscle (0.9–1.0 g) was incubated at 37 °C in 1.5 ml of Krebs–Ringer buffer, pH 7.4, containing 6 mM glucose and 10 μCi of [U-^{14}C]D-glucose with either insulin or NO at various concentrations (in 0.9% NaCl) in the presence or absence of NAME. Glucose oxidation was determined as described elsewhere (*18*).

Determination of Membrane Tyrosine Kinase Activity. Membrane from mice epitrochlearis muscle was prepared, as described (*10*). Typically, 50 μg of epitrochlearis muscle or human RBC membrane (*14*) was solubilized by suspending the membrane preparation in 10 mM Tris-HCl buffer, pH 7.4, containing 0.05% Triton X-100, 0.3 M sucrose (final concentration), 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. After the detergent was added, the membrane suspension was incubated at 0 °C for 30 min with occasional gentle shaking. The detergent-treated membrane suspension was clarified by centrifugation at 20 000 × *g* for 30 min at 0 °C. Protein tyrosine kinase from the solubilized membrane preparation was immunoprecipitated and adsorbed on protein A–Sepharose 4B by using monoclonal anti-phosphotyrosine antibody (1:200 dilution) as described (*19*). The precipitates were collected by centrifugation at 10 000 × *g* for 5 min at 4 °C and washed twice with 0.05% Triton X-100. The precipitates were incubated with either various concentrations of insulin (0–400 μU/ml) or NO (0–0.2 nmol/ml) in the presence or absence of 10 μM NAME at 23 °C for 30–45 min. The protein tyrosine kinase activity was assayed in a final volume of 100 μl consisting of 20 mM HEPES buffer, pH 7.4, 10 mM thioglycerol, 2 mM $MnCl_2$, 10 μM $ZnCl_2$, 8 mM $MgCl_2$, and 0.02% Triton X-100 (v/v), and substrates Glu^{80}-Tyr^{20} copolymer (0.1 mg/ml) in the presence of 10 μM [γ-^{32}P]ATP (4000–6000 cpm/pmol). The phosphorylation of the substrate (Glu^{80}-Tyr^{20}) was determined as described (*7*).

Assay of PI 3-Kinase. Typically, 50 μg of either mice epitrochlearis muscle or RBC membrane preparation was solubilized in 0.01% Triton X-l00, and PI 3-kinase was immunoprecipitated by using antibody to PI 3-kinase and protein A–Sepharose, as described (*20*). The precipitate, resuspended in 20 mM HEPES buffer, pH 7.4, and 10 mM $MgCl_2$, was treated with various concentrations of insulin or NO in 0.9% NaCl in the presence or absence of 10 μM NAME for 60 min at 23 °C. The suspension was then treated with 10 μM (10 μCi) of [γ-^{32}P]ATP and 200 μg of phosphatidylinositol and incubated for 30 min at 23 °C. The reaction mixture was sonicated, after which the reaction was terminated by adding 1 M HCl followed by an equivolume solution of chloroform:methanol. The organic phase was then separated by centrifugation. PI 3-kinase was isolated by thin-layer chromatography and HPLC; PI 3-kinase was quantitated by determining its radioactivity (*20*).

Induction of Diabetes in Mice and Determination of Blood Glucose. Diabetes was induced in mice with alloxan, as described (*21*). Blood glucose concentrations were determined with glucose oxidase strips from Boehinger-Mannheim. The mice were considered to be diabetic when their blood glucose values after an overnight fast were 250–300 mg/dl; the normal range for the control, nondiabetic mice was 70–80 mg/dl. Two hours after feeding ad libitum, the concentrations of blood glucose in the diabetic mice increased to 400–450 mg/dl.

Preparation of Nitric Oxide. The NO used in this study was prepared and purified as described previously (*22*). Typically, 20 g of copper fillings was treated with 30 ml of dilute HNO_3 in a 250-ml borosilicate round-bottom flask fitted with a delivery tube. The evolving gas (NO) was washed by passing sequentially through 2% NaOH and O_2-free cold water. The gas was then bubbled into 1 ml of 0.9% NaCl solution at 0 °C for at least 30 min in an inert atmosphere of N_2. Before use in the preparation of NO, the water was boiled to expel dissolved O_2, then cooled and kept under N_2 in airtight containers. Also, the HNO_3 was flushed with a dry stream of nitrogen. Mass spectroscopic and gas chromatographic analysis of NO showed that the preparation was >99% pure.

The NO in saline was kept under N_2 at 0 °C and used within 15 min after preparation; its concentration in saline was determined by the oxyhemoglobin method (*15*) as described above. For routine experiments, NO was diluted in O_2-free 0.9% NaCl and also taken up in plastic syringes for injection into the tail vein of mice.

RESULTS

Effect of Insulin on the Formation of NO in Various Tissues and the Inhibition of NO Synthesis by NAME. Incubation of whole tissue from intestine, kidney, liver, and skeletal muscle

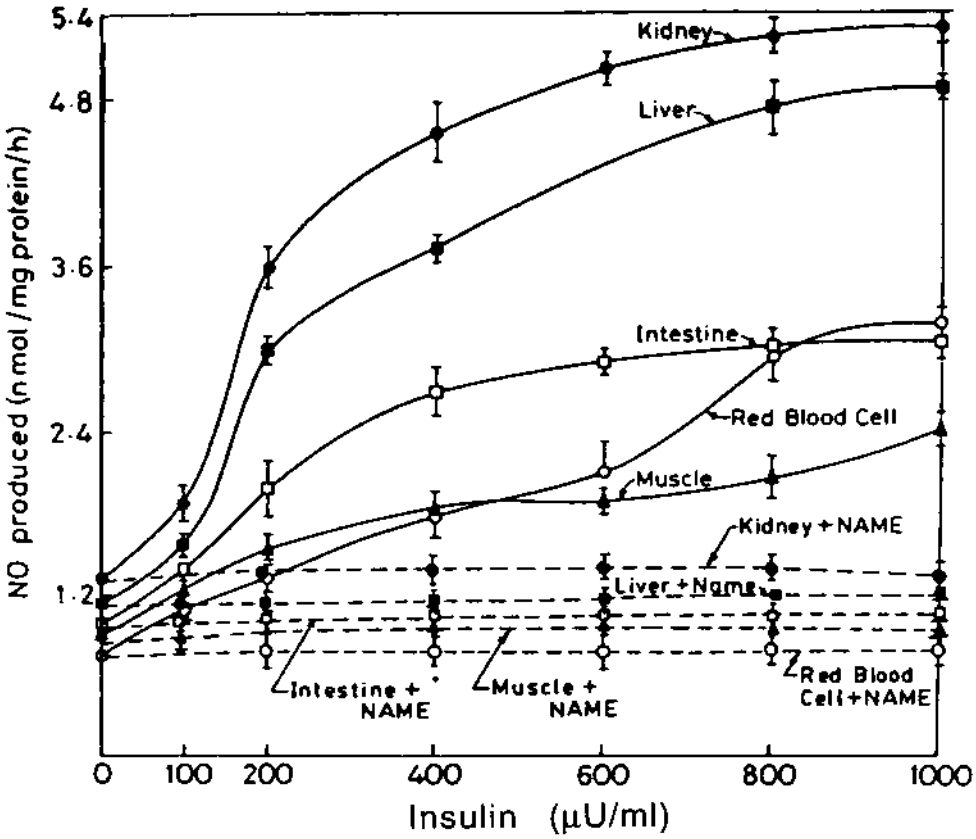

Figure 1. Production of nitric oxide (NO) in various tissues stimulated by insulin. Tissue samples (3–10 mg of protein) from albino mice and human RBCs (10^8 cells/ml) from healthy donors were incubated with various concentrations of insulin. After incubation, the amount of NO in the supernatant was determined. The solid lines represent the production of NO in the presence of insulin. The broken lines represent the production of NO in the presence of insulin + NAME. Each point represents mean ± SD of at least six separate experiments using different tissues from six different animals, or RBCs from six normal volunteers (three men and three women).

from mice and RBC from healthy human volunteers with increasing concentrations of insulin resulted in the increased production of NO by these tissues (Fig. 1). The amount of NO synthesized by insulin-activation in different tissues varied. Addition of 200 μU/ml insulin to the incubation mixture of kidney tissue resulted in the production of 3.6 nmol NO per milligram of protein per hour. The production of NO in RBC membrane was 1.32 nmol NO mg^{-1} protein h^{-1} at 37 °C. Addition of 10 μM NAME, a competitive inhibitor of NOS (*24*), to the incubation mixture completely blocked the insulin-activated production of NO in all tissues. The time course of insulin-stimulated NO production in these tissues varied considerably. At 37 °C, the insulin-stimulated rate of NO production for RBC membrane was linear between 30 and 90 min, whereas the hormone-activated increase of NO production in tissues from heart, liver, kidney, leg muscle, and intestine was linear from 60 to 120 min.

Stimulation of NOS Activity by Insulin in Various Tissue Homogenates and in Purified RBC Membrane. The inhibition by NAME of the insulin-activated stimulation of NO production in various tissues indicated that the increased production of NO by insulin was probably the result of stimulation of NOS activity in these tissues. To determine whether the hormone also stimulates NOS activity in cell lysates, various concentrations of insulin were added to the reaction mixtures containing homogenates of the aforementioned tissues with L-arginine as the substrate. In whole tissues, the data indicated that adding increasing concentrations of insulin resulted in increased synthesis of NO (Fig. 1). Although the dose–response curve of the insulin effect demonstrated considerable variation in the stimulation of NOS activity in various tissue homogenates, in all tissues the addition of 10 μM NAME to the reaction mixture completely inhibited the insulin-activated stimulation of NO synthesis. Furthermore, only the particulate fraction of the homogenate prepared by ultracentrifugation of the tissue homogenate contained the insulin-activatable NOS activity. There was no detectable insulin-activatable NOS activity in the soluble cytosolic fraction of the homogenate. The basal NOS activities of the particulate fractions from the heart, liver, kidney, muscle, and RBC

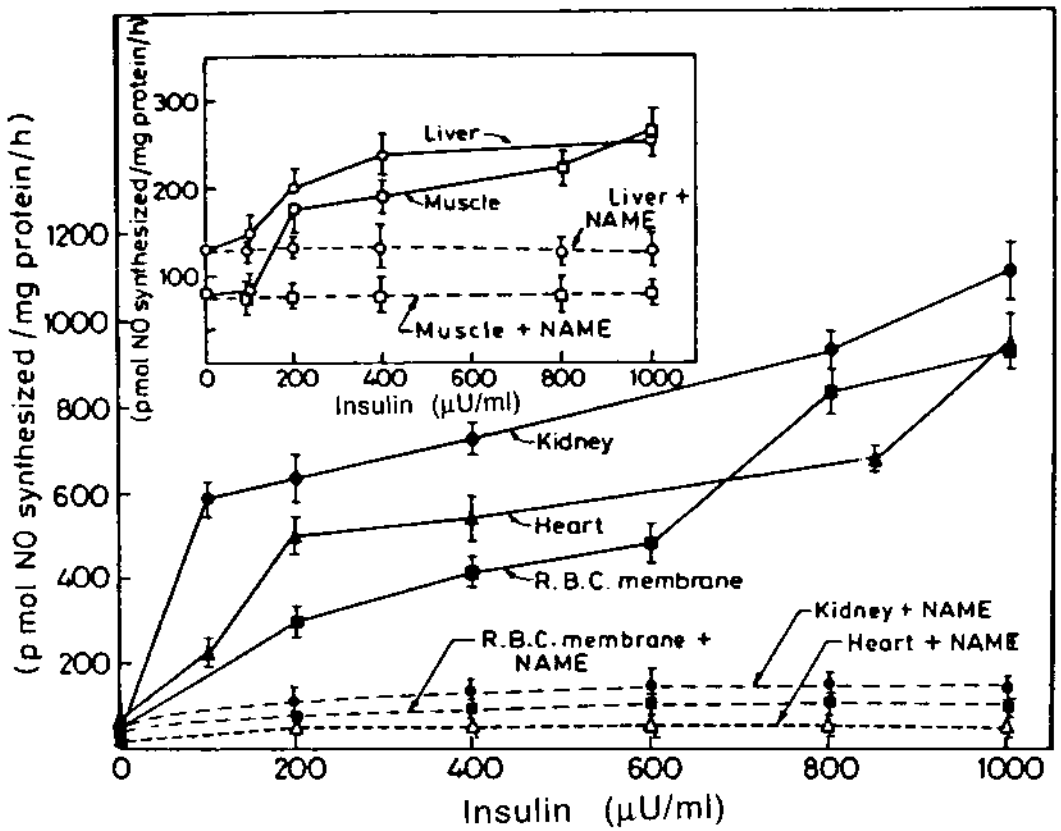

Figure 2. Stimulation of NO synthesis in various tissue homogenates and RBC membranes by insulin. Homogenates from various tissues were centrifuged at 60 000 × *g* at 0 °C for 30 min. The supernatant and the particulate fractions were separated. NOS activity was determined in both fractions. The effect of NAME on the synthesis of NO was determined by adding 10 μM NAME to the reaction mixtures under identical conditions. Each point represents the mean ± SD of at least 10 different experiments, each in triplicate. The solid lines represent the synthesis of NO in the presence of insulin. The broken lines represent the synthesis of NO in the presence of insulin + NAME in the different homogenates as indicated.

membrane were 0.075 ± 0.015, 0.04 ± 0.13, 0.0125 ± 0.023, 0.08 ± 0.019, and 0.04 ± 0.01 nmol of NO formed mg^{-1} protein h^{-1} (n = 3), respectively; when the same particulate fractions were treated with 200 μU of insulin per milliliter, these increased to 0.475 ± 0.022, 0.657 ± 0.03, 0.200 ± 0.055, 0.300 ± 0.018, and 0.500 ± 0.0.046 nmol of NO formed mg^{-1} protein h^{-1}, respectively. In contrast, the NOS activities of the supernatant fractions from heart, kidney, liver, muscle, and RBC membranes were 0.089 ± 0.033, 0.069 ± 0.013, 0.07 ± 0.025, 0.04 ± 0.01, and 0.055 ± 0.018 nmol NO of formed mg^{-1} protein h^{-1} (n = 3), respectively, and remained essentially unchanged when treated with insulin under identical conditions: 0.09 ± 0.027, 0.073 ± 0.023, 0.082 ± 0.019, 0.054 ± 0.025, 0.065 ± 0.018 nmol of NO formed mg^{-1} protein h^{-1}, respectively (Fig. 2).

Effect of Ca^{2+} on the Insulin-Stimulated NOS Activity. When intact tissues or tissue homogenates were incubated in Ca^{2+}-free Krebs buffer under the conditions described above, the stimulation of NO synthesis in the presence of insulin was minimal (0.095 ± 0.01 nmol of NO formed mg^{-1} protein h^{-1}). Furthermore, the insulin-activated formation of NO increased with increasing concentration of Ca^{2+} (from 0 to 2.0 mM); at 1.5–2.0 mM Ca^{2+} ion concentration, insulin-activated NO was maximally increased by fivefold over the control. Adding Ca^{2+} > 2.0 mM inhibited the insulin-stimulated NO formation. Addition of EDTA (1.0 mM final concentration) to the reaction mixture completely inhibited the insulin-stimulated NO formation.

Binding of Insulin to Purified Red Blood Cell Membranes and the Activation of NOS. In light of these results, which suggested that the insulin-activatable NOS may be a membrane-bound constitutive enzyme, human RBC membranes were purified and studied. Incubation only of purified RBC membrane (not the membrane-free RBC lysate) with insulin-stimulated synthesis of NO, which was directly related to the binding of insulin to the RBC membranes (Fig. 3). The concentration-dependent binding of insulin was analyzed by Scatchard plot (*17*). Computer analysis of the data showed the presence of one high-affinity/low-capacity insulin binding site population

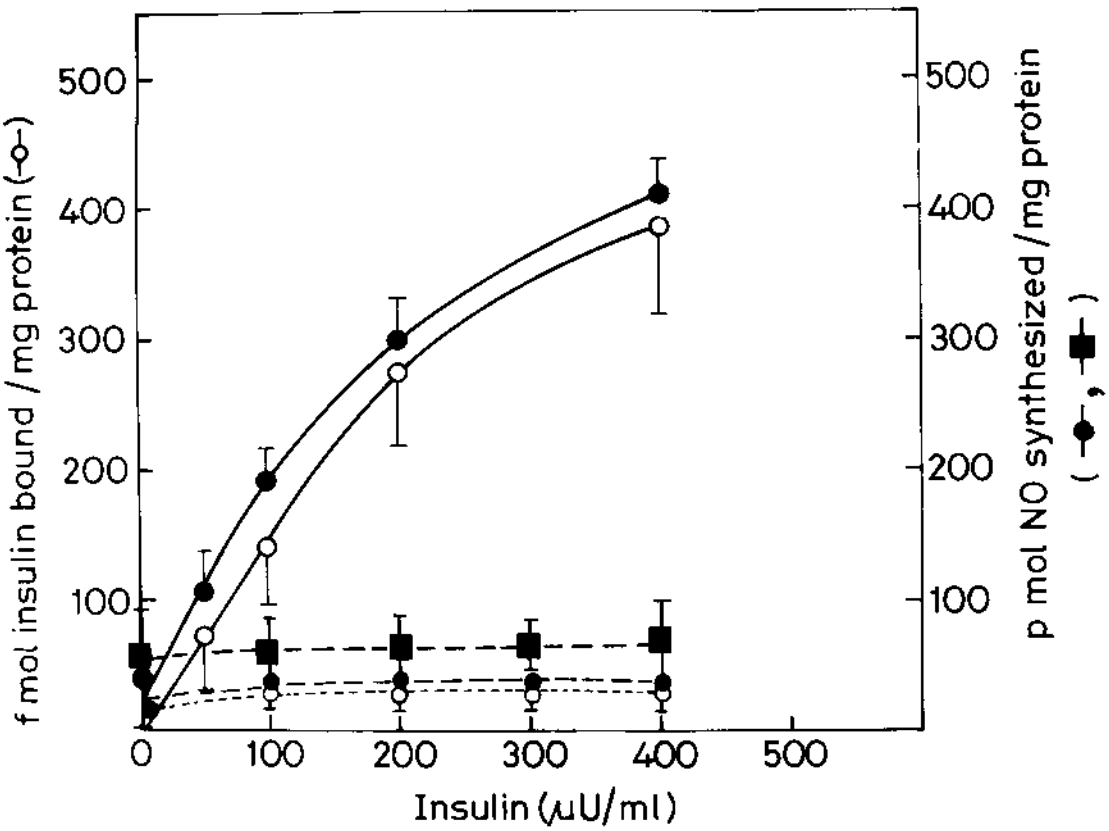

Figure 3. Binding of insulin to human RBC membranes and the associated synthesis of NO. Human RBC membrane preparation was incubated with various concentrations of insulin for 60 min at 23 °C. The specific binding of insulin to the membrane preparation was determined by using ^{125}I-labeled insulin (*14*). In parallel experiments unlabeled insulin was used, and the simultaneous synthesis of NO was determined. Results shown are expressed as mean ± SD of at least six experiments, each in triplicate. Open circles represent binding of insulin; closed circles represent NO synthesis. Solid squares represent synthesis of NO by the cytosolic fraction in the presence of various concentrations of insulin. RBC membranes were also incubated with purified anti-insulin receptor antibody. Open circles and broken lines represent binding of insulin; solid circles and broken lines represent insulin-induced NO synthesis in RBC membrane.

($K_{d_1} = 2.20 \times 10^{-9}$ M, $n_1 = 290$ fmol/mg protein) and one low-affinity/high-capacity insulin binding site population ($K_{d_2} = 0.801 \times 10^{-6}$ M, and $n_2 = 40$ pmol/mg protein). Addition of 50–100 μg of purified anti-insulin antibody to the purified RBC membranes inhibited both the binding of the hormone to its receptors on the membrane and the production of NO because of the hormone–receptor interaction (Fig. 3).

Effect of Insulin on the K_m *and* V_{max} *of NOS Activities in Various Membrane Preparations.* A Lineweaver–Burk plot of the NOS activity of the RBC membranes demonstrated that the stimulation of enzyme activity by insulin was the result of a decrease of the K_m of L-arginine from 1.508 ± 0.0113 to 0.805 ± 0.040 μM ($P = 0.0019, n = 10$) when compared with the control, and a simultaneous increase of V_{max} from 24.4 ± 0.384 to 56.4 ± 0.329 pmol of NO synthesized mg^{-1} protein min^{-1} ($P = 0.00008$). Similar changes of K_m and V_{max} of NOS also occurred in the particulate fraction of the intestine tissue treated with insulin. In the intestinal homogenate preparation, the K_m of NOS decreased from 1.488 ± 0.25 to 0.969 ± 0.014 μM, and the V_{max} increased from 5.6 ± 0.424 to 10.923 ± 0.411 pmol of NO formed mg^{-1} protein min^{-1}, respectively, when the same sample was treated with insulin ($n = 6$). Addition of 10 μM NAME to the reaction mixture containing various concentrations of L-arginine (added for the determination of K_m and V_{max}) completely inhibited NO production.

Effect of NO on Glucose Transport, on Glucose Oxidation in Mice Epitrochlearis Muscle, and on Tyrosine Kinase and PI 3-Kinase Activity in the Muscle Membrane Preparation. Because treating both the whole tissue and their homogenates with insulin stimulated the formation of NO, we tested the possibility that NO itself might have an insulin-mimetic effect on carbohydrate metabolism. The effects of NO and insulin on glucose transport activity and on glucose oxidation in mice epitrochlearis muscle were compared (*18*). Our data indicated

that NO mimicked the stimulatory effect of insulin in glucose transport activity and glucose oxidation (Fig. 4a and b). Furthermore, addition of NAME to the incubation mixture, which blocked the insulin-activated synthesis of NO in this tissue, also completely blocked these stimulatory effects of insulin.

Because NO mimicked the insulin effects on carbohydrate metabolism in vitro in epitrochlearis muscle, the effect of NO on the activation of tyrosine kinase and PI 3-kinase in the muscle membrane was determined. Similar to the effects of insulin, treating the muscle membrane fraction with NO, activated both the receptor tyrosine kinase and PI 3-kinase to a comparable extent, regardless of the presence or absence of NAME (Fig. 4c and d). Given that the inhibition of insulin-activated NO formation by NAME completely inhibited the insulin effect on carbohydrate metabolism (Fig. 4a and b), whereas the presence of NAME had no effect on the insulin mimetic activity of NO on the tyrosine and IP-3 kinases activation (Fig. 4c and d), this indicated that insulin-activated formation of NO was necessary for expression of the insulin effect and that the NO thus generated was capable of producing an insulin effect.

Control of Hyperglycemia by Insulin and Production of NO in Vivo. To determine whether NO had a hypoglycemic effect in vivo, various concentrations of NO or insulin were administered to diabetic mice. The postprandial (0 h) concentration of glucose (417.5 ± 6.89 mg/dl, mean ± SD, $n = 6$) had by 2 h after injection decreased significantly in those mice injected with either insulin (200 μU/g body wt) or NO (0.6 nmol/g body wt), to 138 ± 7.5 and 125.83 mg/dl ($P < 0.0001$, $n = 6$), respectively, in comparison with the control diabetic mice (264.17 mg/dl). Injection of sodium nitroprusside (600 pmol/g body wt in 0.09% NaCl), which is an NO donor in vivo, also decreased the blood sugar concentration, from 234 ± 17 to 130 ± 0.95 mg/dl ($P < 0.00001$, $n = 12$), at 2 h. In contrast, injection of NAME at 10 μM/g body wt into diabetic mice 15 min before the administration of insulin (200 μU/g body wt) effectively blocked the anticipated decrease in blood glucose in these animals compared with the control animals (264.17 ± 11.14 vs. 252.50 ± 5.24 mg/dl, $P = 0.078$, $n = 6$) (Fig. 5a). Administration of NO in the presence of NAME, however, did not prevent the decrease in blood glucose (126.67 ± 8.16 mg/dl, $P < 0.0001$), indicating that NAME per se did not modify the hypoglycemic effect of NO (Fig. 5b). These data indicating the failure of insulin to reduce the blood glucose concentration in diabetic mice in the presence of NAME are related to the complete inhibition of insulin-activated NO production. Injection of NAME alone failed to produce any detectable effect on blood glucose content in these diabetic mice. In contrast, administration of 10 μM NAME to nondiabetic mice inhibited the rate of decrease in blood glucose by ~30% compared with the controls, despite the presence of preformed NO (200 ± 50 pmol/ml) in the blood at the time of injection of NAME.

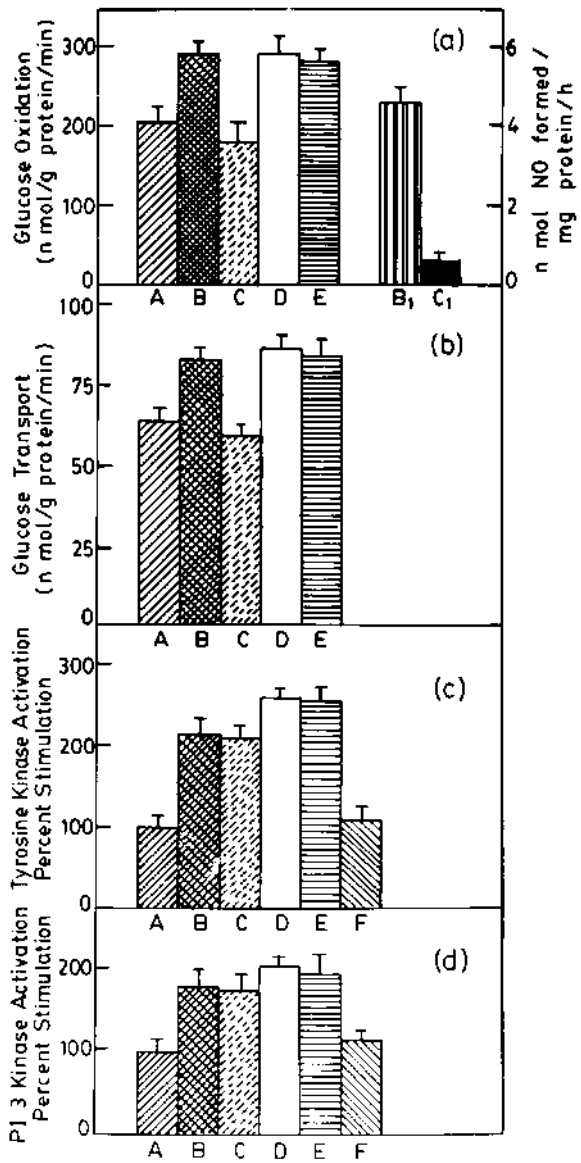

Figure 4. NO mimics the effect of insulin on glucose transportation and oxidation in mice epitrochlearis muscle and on insulin receptor tyrosine kinase and PI 3-kinase of the membrane. (a) Glucose oxidation and NO production by insulin. Glucose oxidation in mice epitrochlearis muscle was determined by using 1-^{14}C-glucose (*29*); the muscle preparation (1–2 g per sample) was treated with either insulin, 200 μU/ml, or NO, 2 nmol/ml, in the presence or absence of 10 μM NAME, as indicated. Bar A, control; bars B and C, treatment of muscle with insulin and insulin + NAME, respectively; bars D and E, treatment of muscle with NO and NO + NAME, respectively. Bars B_1 and C_1, NO formation in the presence of insulin and insulin + NAME, respectively. (b) Glucose transport activity was measured by using 3-*O*-^{14}C-methyl glucose (*17*). Bars A–E, control and respective treatments as in (a). (c) Insulin-induced tyrosine kinase activity was determined as described in the text. Bars A–E, control and respective treatments as in (a); bar F, NAME. (d) The PI 3-kinase activity of the membrane preparation was determined as described (*16*). Bars A–F as in (c).

DISCUSSION

Our results provide a novel demonstration that insulin is capable of activating a membrane-bound NOS in different tissues

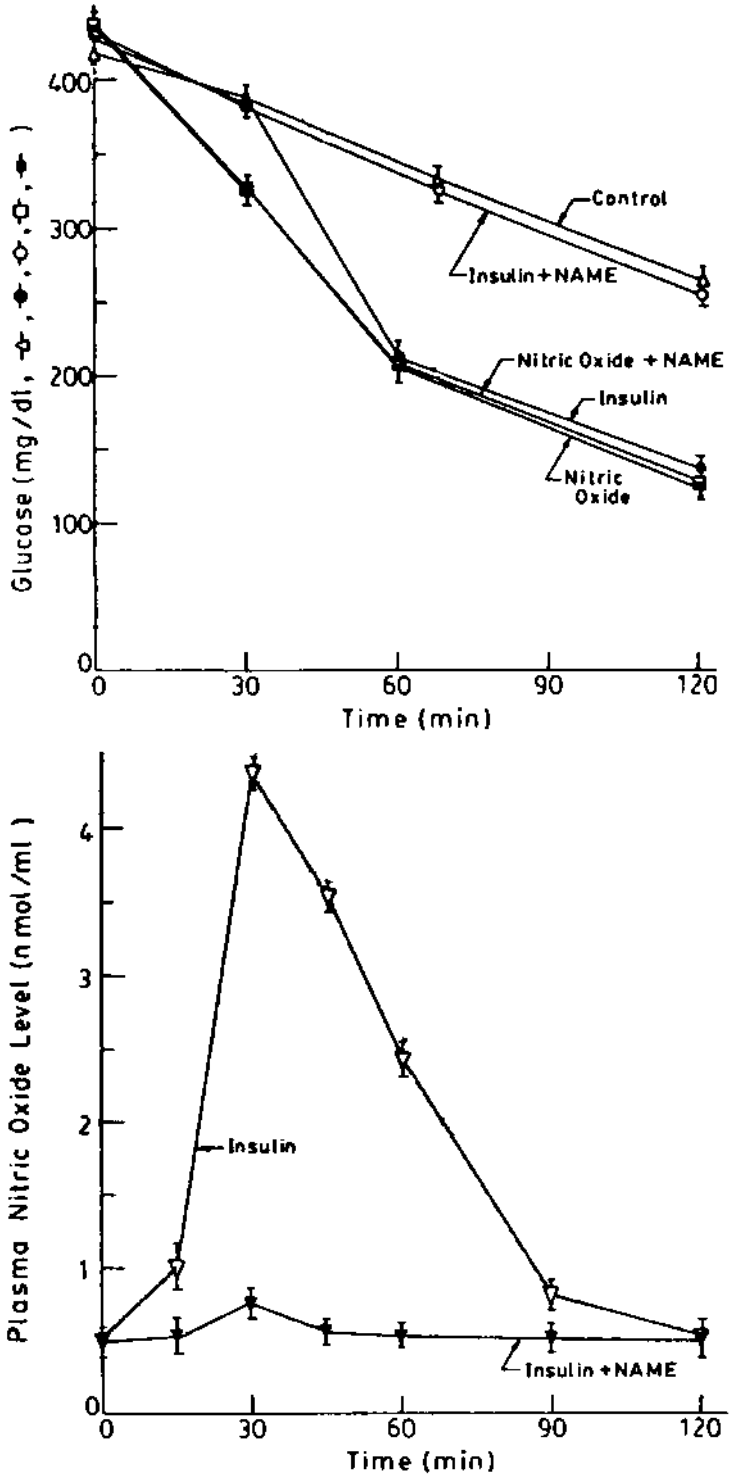

Figure 5. (a) Effect of insulin or NO on blood glucose concentrations in diabetic mice. Diabetes-induced mice were treated with either insulin, 200 μU/g body wt, or NO, 600 pmol/g body wt (NO in 0.9% NaCl). Insulin was administered by injection in the leg muscle; NO was given intravenously. At the times shown, blood glucose concentrations were determined. In control experiments, an equal volume of saline was similarly injected. In parallel experiments the mice were injected with 10 μM NAME in 0.9% NaCl before they were treated with insulin (as described above) and the blood glucose contents were determined. Open triangles and solid circles represent plasma glucose concentrations at various times in control and insulin-treated diabetic mice, respectively. Open circles represent the effect of NAME + insulin at the same time. Solid squares and open squares represent the blood glucose concentrations at various times after NO or NO + NAME was injected in diabetic mice, respectively. (b) Open inverted triangles and solid inverted triangles represent plasma NO concentrations at various times after the injection of insulin or insulin + NAME, respectively. Results are the mean ± SD of six experiments in six diabetic mice.

and that the NO thus formed produces insulin-like effects in the absence of the hormone. In addition, our data demonstrate that RBCs in the circulation could be the major cell system for the insulin-stimulated NO formation. Although insulin-treated human endothelial cells from the umbilical cord vein when treated with insulin also produced NO, the amount of insulin-stimulated NO produced by RBCs was at least four- to fivefold greater than in the endothelial cells (unpublished data). Furthermore, although the presence of NOS in the soluble cytosolic fraction is well known (*23*), the cytosolic NOS could not be stimulated by insulin, and the biochemical characteristics of the membrane-bound insulin-activatable NOS differed from those of the cytosolic NOS. For example, the synthesis of NO from L-arginine by insulin-activated NOS, unlike that by the soluble cytosolic NOS, was not dependent on the addition of ATP and NADPH; however, the presence of Ca^{2+} was essential for insulin-activated formation of NO. Thus the insulin-activatable membrane NOS as described here is different from the previously reported cytosolic NOS (*4*).

Although details of the activation of the membrane-bound NOS by insulin and the mechanism of reaction remain to be determined, our results indicate that the stimulation of NOS activity by insulin was the consequence of the increase of the reaction velocity V_{max} with the simultaneous decrease of K_m. These values are in the physiologic range of the tissue concentration of L-arginine. The activation of NOS by insulin in purified RBC membranes, which do not synthesize proteins, and the rate of NOS activation by the hormone indicate that the insulin-activated NOS is probably a membrane-bound constitutive enzyme, the activation of which is directly related to the binding of the hormone to its specific binding sites on the membrane surface. Furthermore, addition of anti-insulin receptor antibody to the RBC suspension, which blocked the binding of insulin to its receptors on the cell membrane, also inhibited activation of NOS by insulin. These results demonstrate that the insulin-stimulated activation of NOS on the RBC membrane was the consequence of the binding of the hormone to its receptors.

The results herein also demonstrate that insulin-stimulated activation of tyrosine kinase or PI 3-kinase was unaffected in the presence of NAME. However, the addition of NAME to the reaction mixture totally inhibited both the NO synthesis and the insulin-activated stimulation of carbohydrate metabolism in vitro and in vivo, indicating an essential role of NO in insulin action. Furthermore, these results indicate that the activation of tyrosine kinase and PI 3-kinase by insulin does not necessarily result in the obligate transduction of the insulin effect on carbohydrate metabolism, although it does appear that the insulin-activated production of NO is essential. Inhibition of PI 3-kinase by wortmannin or of tyrosine kinase by genistein reportedly inhibits the insulin-induced NO production in human umbilical cord vein endothelial cells (*24*). Recently we have found that removal of either tyrosine kinase or PI 3-kinase from the solubilized membrane preparation by using monoclonal antibodies against these enzymes completely inhibited any insulin-activated NO synthesis, indicating an essential role of these enzymes in the activation of NOS (unpublished results). Nevertheless, the effect of NAME on insulin-activated NO formation and the ensuing stimulation of carbohydrate metabolism demonstrated that generation of NO was essential for the insulin-signaling pathway (*19*, *20*).

The results as reported by these investigators (*19*, *20*), together with our data here, may indicate a possible convergence of the biochemical pathways involving both tyrosine kinase and PI 3-kinase, in the downstream synthesis of NO as the "second messenger" through activation of NOS in the transduction of the insulin message, at least with regard to the message for carbohydrate metabolism. Additionally, NOS may be postulated to be a substrate for the insulin receptor tyrosine kinase. Recently, we found that the purified membrane-bound, insulin-activatable NOS is phosphorylated by the purified insulin receptor in the presence of insulin, an effect that can be blocked by an anti-insulin receptor antibody (unpublished data). Furthermore, the effect of anti-insulin receptor antibody on the blocking of the binding of insulin to its receptors and the consequent inhibition of NO production by insulin indicate that the synthesis of NO by insulin is a postreceptor interaction phenomenon essential for the insulin effect. Additionally, the insulin-mimetic effect of NO and the inhibition of insulin effect by NAME in diabetic mice, and the observed effect of the inhibitor (NAME) on the rate of decrease of blood glucose in nondiabetic mice, suggest a critically important role of NO synthesis in the control of hyperglycemia by insulin. Furthermore, not only was NO capable of activating tyrosine kinase or PI 3-kinase but also the "messenger" NO could activate NOS in the absence of either tyrosine kinase or PI 3-kinase (or both) in the NOS system (unpublished results), whereby NO would probably amplify its own generation by "feedback" activation of NOS and, consequently, would amplify the effect of insulin itself in the system.

It has been reported previously that NO can stimulate glucose metabolism in tissues (*25*) and that insulin treatment of platelets, cells that do not require insulin for glucose metabolism, results in the increased production of NO (*26*). However, it has not been previously appreciated that the production of NO as a result of the activation of NOS by insulin is actually an essential step—beyond the activation of both PI 3-and tyrosine kinases by insulin—in the transduction of messages that affect glycemic control.

Our results suggest that NO might be the second messenger molecule for the stimulatory effect of insulin in carbohydrate metabolism. This postulate is supported by the following observations.

1. Exposure of various tissues to insulin resulted in the synthesis of NO because of the activation of NOS through the binding of the hormone to its receptors.
2. NO, the product thus formed, was capable of mimicking one of the most important effects of insulin, that is, the stimulation of carbohydrate metabolism both in vitro and

in vivo. NO, the proposed second messenger of insulin, was also capable of activating PI 3- and tyrosine kinases, similar to the effect of insulin.

3. The inhibition of insulin-activated NO synthesis simultaneously inhibited the insulin-lowering effect on serum glucose concentration; however, the activation of both PI 3- and tyrosine kinases by insulin was unaffected.

Because of its effects on various biological events, NO has been previously proposed to be a messenger molecule (*27*, *28*). However, in contrast to any previous investigations, NO has not been designated the messenger of any particular agent or hormone action. Our results herein strongly indicate that NO may be the second messenger for insulin action on carbohydrate metabolism and possibly also serves a role with regard to other metabolic or ionic actions.

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