Effects of Acute Euglycemic Hyperinsulinemia on Urinary Nitrite/Nitrate Excretion and Plasma Endothelin-1 Levels in Men With Essential Hypertension and Normotensive Controls

Andrzej Surdacki, Michal Nowicki, Joerg Sandmann, Dimitrios Tsikas, Olga Kruszelnicka-Kwiatkowska, Franciszek Kokot, Jacek S. Dubiel, and Juergen C. Froelich

Insulin stimulates the production of endothelin-1 (ET-1) and nitric oxide (NO) by isolated endothelial cells. Additionally, insulin-dependent glucose transport and insulin-mediated NO production partially share common elements in signal transduction. There are discordant data on plasma ET-1 levels during acute euglycemic systemic hyperinsulinemia in normotensive men and men with essential hypertension (EH) (known to be insulin-resistant), as well as on the relations between insulin sensitivity and vascular function. Our aim was to assess the response of approximate measures of whole-body generation of NO and ET-1 to acute euglycemic hyperinsulinemia in EH patients and controls. We studied 17 newly diagnosed untreated men with uncomplicated EH and 10 normotensive controls. Plasma ET-1 and urinary excretion of nitrite plus nitrate, stable NO metabolites (Uno_x), were measured before and during a 3-hour hyperinsulinemic-euglycemic clamp. Both in hypertensives and normotensives, plasma ET-1 levels were reduced after 2 hours of the clamp (EH: baseline, 3.1 ± 1.9 pg/mL; 2 hours, 1.9 ± 1.2 pg/mL, $P = .04$ *v* baseline; controls: baseline, 4.2 ± 2.6 pg/mL; 2 hours, 2.8 ± 1.4 pg/mL, $P = .04$ *v* baseline). No significant changes in Uno_x during the clamp were observed. Changes in Uno_x during the clamp (ΔUno_x) and differences in plasma ET-1 measured before the end and before the beginning of the clamp (ΔET-1) were correlated in the controls ($r = .75$, $P = .01$) but not in EH ($r = -.01$, $P = .97$). No parameter of glucose metabolism correlated with basal Uno_x, basal plasma ET-1, ΔUno_x, and ΔET-1, whether absolute or percent values, in either group. Thus, acute euglycemic hyperinsulinemia produces a decrease in plasma ET-1 in both EH patients and controls. The lack of correlation between ΔUno_x and ΔET-1 under these conditions in EH may suggest an impairment of systems governing interactions between the NO-dependent pathway and ET-1. In addition, insulin actions on glucose metabolism and on the endothelial mediators appear dissociated.

INSULIN STIMULATES the production of both endothelin-1 (ET-1)[1,2] and nitric oxide (NO)[3] by isolated human endothelial cells. However, there are contradictory reports on the changes in plasma ET-1 levels during acute euglycemic systemic hyperinsulinemia in both normotensive individuals and those with essential hypertension (EH). In contrast to Piatti et al[4] and Wolpert et al,[5] who observed an increase in circulating ET-1 in normotensives and hypertensives, respectively, Polderman et al[6] found unchanged plasma ET-1 in healthy men, whereas Seljeflot et al[7] recently reported a decrease in ET-1 during a hyperinsulinemic-euglycemic clamp in EH subjects. In addition, although endothelial NO synthesis within limbs is also stimulated in such conditions,[8,9] Polderman et al[6] have found that indirect indices of whole-body NO formation, namely increases in plasma cyclic guanosine 3′,5′-monophosphate (cGMP) and in the L-citrulline to L-arginine ratio, were observed exclusively in women and were absent in healthy men.

EH is associated with both insulin metabolic resistance (IR)[10] (mainly localized within the skeletal muscle[11]) and a reduced dependence of basal vascular resistance on the NO-dependent pathway.[12,13] Insulin-dependent glucose transport and insulin-mediated NO production partially share common elements in signal transduction.[3] A hypothesis has been proposed suggesting the coupling of glucose metabolism and NO-dependent dilation in skeletal muscle.[14] However, there are discordant data on whether the magnitude of IR is related to the degree of impairment in the ability of hyperinsulinemia to produce vasodilation,[15-17] as well as to the basal activity of the L-arginine–NO pathway within the forearm.[18-20] Moreover, in EH, the capability of local hyperinsulinemia to facilitate the dilatory effects of acetylcholine in forearm vessels was intact, yet this influence was mediated not via a NO-dependent mechanism (as found in normotensives) but through a NO-independent, ouabain-inhibitable pathway.[21]

Our aim was to assess the response of approximate measures of whole-body generation of both NO and ET-1 to acute euglycemic hyperinsulinemia in untreated male subjects with mild uncomplicated EH and normotensive controls. In addition, we estimated the relationship between insulin metabolic sensitivity and endogenous NO and ET-1 production.

SUBJECTS AND METHODS

Patients

We studied 17 newly diagnosed untreated male subjects with mild uncomplicated EH, a positive family history of EH, a body mass index (BMI) of 30 kg/m^2 or less, a total cholesterol level of 6.4 mmol/L or less, and normal creatinine clearance and without clinical or biochemical evidence of other coexistent disease. The control group consisted of 10 healthy males with no family history of EH, matched for age, BMI, and cholesterol without evidence of any disorders. We excluded subjects with an inflammatory process (as assessed by medical history and assay for C-reactive protein), a stimulus for inducible NO synthase expression.[22]

From the Institute of Cardiology, Jagiellonian University, Cracow; Department of Nephrology, Silesian University School of Medicine, Katowice, Poland; and Department of Clinical Pharmacology, Hannover Medical School, Hannover, Germany.

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Address reprint requests to Andrzej Surdacki, MD, 17 Kopernika St, 31-501 Cracow, Poland.

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Protocol

The protocol was approved by the ethics committee of our institution, and informed consent was obtained from all subjects. The patients were instructed to avoid nitrate-rich food and to refrain from consuming beverages containing alcohol or caffeine for 48 hours prior to the procedure. After an overnight fast of 12 to 14 hours, they were asked to empty their bladder and subsequently remained at rest for 3 hours. Thereafter, they passed urine, blood samples were taken, and blood pressure and heart rate were measured with an automatic sphygmomanometer. Although, in accordance with the plasma half-life of nitrate of about 6.5 hours,[23] a much longer fasting period might have been considered, the urinary excretion rate of nitrite plus nitrate (Uno_x) after an overnight fast was shown to be independent of nitrate intake during the preceding days.[24]

A hyperinsulinemic-euglycemic clamp was performed according to the method of DeFronzo et al[25] using a 3-hour primed infusion of 359 pmol/m^2/min soluble insulin. Glycemia was kept constant by infusion of 20% glucose. Immediately after termination of the clamp, the subjects provided the second urine sample.

Arterialized venous blood for ET-1 and insulin assay was taken immediately before beginning the insulin infusion, after 2 hours, and just before termination of the clamp. Blood samples for ET-1 assay were drawn into prechilled tubes containing EDTA and aprotinin and immediately centrifuged at 4°C, and the plasma was kept frozen at −20°C until radioimmunoassay.

Urine samples were diluted with 2-isopropanol at a final concentration of 10% to prevent bacterial activity[26] and frozen at −20°C until assays for nitrite plus nitrate (NO_x) and creatinine. NOx was determined using gas chromatography–mass spectrometry.[27] Uno_x was corrected for urinary creatinine and expressed as micromoles per millimole of creatinine to eliminate the effect of variability in renal function.[28] The creatinine level was measured with the alkaline picric acid method.

The whole-body glucose disposal rate (GDR) was calculated from the glucose infusion rate per minute during the last 60 minutes of the clamp and expressed as micromoles per kilogram per minute. An index of insulin sensitivity (ISI) was computed as the GDR divided by the mean insulin level (nanomolars) at the last clamp hour.[29]

Statistical Analysis

Results are presented as the mean ± SD. Baseline characteristics of both groups were compared by two-tailed Student's *t* test for unpaired data. ANOVA for repeated measures was performed with either Uno_x or plasma ET-1 as a dependent variable and the clamp procedure as a within-subjects factor. Post hoc comparisons were performed with Tukey's honestly significant difference test. Additionally, Pearson correlation coefficients (*r*) for selected pairs of variables were calculated. A probability level of less than .05 was considered significant.

RESULTS

Apart from blood pressure, the statistically significant differences between EH subjects and controls were the lower high-density lipoprotein (HDL) cholesterol, GDR, and ISI in EH (Table 1). At baseline, Uno_x was reduced in EH (56 ± 17 *v* 79 ± 25 μmol/mmol creatinine, $P = .01$), whereas basal plasma ET-1 levels were similar in both groups (EH *v* controls, 3.1 ± 1.9 *v* 4.2 ± 2.6 pg/mL, $P = .25$). Plasma insulin during the clamp was 884 ± 221 (2 hours) and 864 ± 204 (3 hours) pmol/L in EH men. In the control group, the respective values were 790 ± 132 and 776 ± 160 pmol/L (EH *v* controls, $P > .2$).

In the EH group, plasma ET-1 levels were reduced after 2 hours of the clamp, whereas during the last clamp hour no significant changes were found (baseline, 3.1 ± 1.9 pg/mL; 2 hours, 1.9 ± 1.2 pg/mL, $P = .04$ *v* baseline; 3 hours, 2.6 ± 1.6 pg/mL, $P = .4$ *v* 2 hours and $P = .5$ *v* baseline; Fig 1). A similar time course of plasma ET-1 was found also in the normotensive group (baseline, 4.2 ± 2.6 pg/mL; 2 hours, 2.8 ± 1.4 pg/mL, $P = .04$ *v* baseline; 3 hours, 4.0 ± 2.5 pg/mL, $P = .44$ *v* 2 hours and $P = .65$ *v* baseline). The lack of statistically significant changes in Uno_x during insulin infusion was observed in both groups (EH, 56 ± 17 *v* 65 ± 31 μmol/mmol creatinine before and after the clamp, respectively, $P = .5$; normotensives, 79 ± 25 *v* 83 ± 41 μmol/mmol creatinine, $P = .3$).

Changes in Uno_x (ΔUno_x) during the clamp and the difference in plasma ET-1 measured at the end of and before the clamp (ΔET-1) were correlated in the controls ($r = .75$, $P = .01$) but not in EH subjects ($r = -.01$, $P = .97$). There was no correlation between the GDR or ISI and any of the following variables in either group: baseline Uno_x, baseline plasma ET-1, ΔUno_x, and ΔET-1, whether absolute or percent values ($P > .25$ in EH and $P > .3$ in controls).

Table 1. Characteristics of EH Patients and Control Subjects

Variable	EH	Control
Age (yr)	39 ± 10	41.5 ± 9
BMI (kg/m^2)	26.3 ± 2.7	26.8 ± 2.4
Creatinine clearance (mL/min)	118.5 ± 15	117.5 ± 19
Systolic blood pressure (mm Hg)	149 ± 15†	125 ± 14
Diastolic blood pressure (mm Hg)	97 ± 12†	82 ± 8
Heart rate (beats/min)	74 ± 13	67 ± 11
Total cholesterol (mmol/L)	4.4 ± 1.0	4.5 ± 1.1
LDL cholesterol (mmol/L)	3.0 ± 0.9	2.9 ± 0.8
HDL cholesterol (mmol/L)	1.0 ± 0.2†	1.3 ± 0.2
Fasting glucose (mmol/L)	5.2 ± 0.4	5.35 ± 0.4
Fasting insulin (pmol/L)	72 ± 33.5	64 ± 49
GDR (μmol/kg/min)	32.3 ± 10.3*	41.9 ± 9.3
ISI ([μmol/kg/min]/[nmol/L])	39.6 ± 17.8*	55.8 ± 17.2

NOTE. Values are the mean ± SD.
Abbreviation: LDL, low-density lipoprotein.
*$P < .05$ *v* control.
†$P < .01$ *v* control.

DISCUSSION

The decrease in plasma ET-1 after 2 hours of the clamp is in keeping with a recent study by Seljeflot et al,[7] who have shown

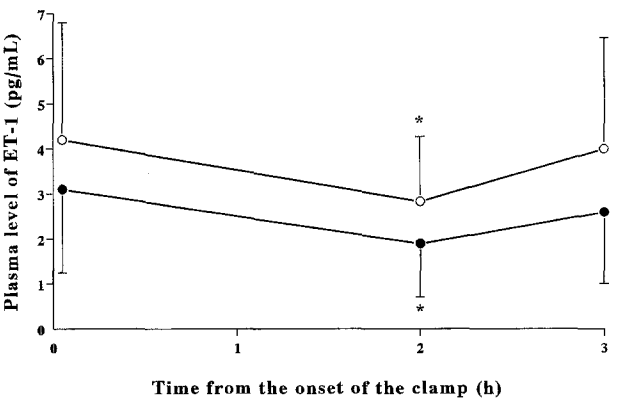

Fig 1. Plasma level of ET-1 in arterialized blood of 17 EH patients (●) and 10 normotensive controls (○) during a 3-hour hyperinsulinemic-euglycemic clamp. *$P < .05$ *v* baseline.

significant decreases in ET-1 during acute 2-hour hyperinsulinemia of similar magnitude in both hyperglycemic and euglycemic conditions in untreated subjects with EH. On the other hand, Polderman et al[6] (hyperinsulinemic-euglycemic clamp) and Leyva et al[2] (intravenous glucose tolerance test) found no significant changes in circulating ET-1 in healthy subjects at insulin levels comparable to ours, in contrast to the report by Piatti et al[4] (euglycemic hyperinsulinemia). In addition, both in normal controls and in non-obese patients with uncomplicated EH and normal glucose tolerance, Ferri et al[30] observed increases in plasma ET-1 in a glucose tolerance test, in contrast to Andronico et al,[31] who found such changes only in EH associated with impaired glucose tolerance. It can be speculated that these discrepancies may be at least partially ascribable to different characteristics of EH patients. Indeed, the blood pressure of our EH patients was closer to the respective values in subjects studied by Seljeflot et al,[7] whereas EH patients who exhibited an increase of ET-1 at hyperinsulinemia had either higher blood pressure[30] or coexistent abnormalities such as severe obesity[5] or impaired glucose tolerance.[31] Nevertheless, this interpretation is insufficient when trying to explain various patterns of the ET-1 response to acute euglycemic[4,6] or hyperglycemic[2,30,31] hyperinsulinemia in healthy individuals.

With regard to a potential mechanism of the decrease in ET-1 under clamp conditions, Seljeflot et al[7] proposed that endothelial NO release evoked by acute hyperinsulinemia might inhibit ET-1 generation, in agreement with previous in vitro[32] and in vivo[33] data suggesting the relevance of this interaction between NO and ET-1. Assuming such a hypothesis, it might have been expected that subjects with a higher ΔUno_x would also exhibit a lower ΔET-1, whereas an opposite relationship was found in healthy men in our study. Following another suggestion proposed by Seljeflot et al[7] (who used a 2-hour clamp technique), we can speculate that the underlying mechanism of the decrease in plasma ET-1 during the first 2 hours of the clamp was a direct effect of vasodilation known to occur in such conditions.[34]

It should be noted that plasma ET-1 has considerable limitations as a measure of endogenous ET-1 production, because it represents only a spillover of ET-1 (secreted in the polar way on the abluminal site of the endothelium[35]) from the vascular wall. This can explain the fact that Leyva et al[2] found a lack of plasma ET-1 changes during acute hyperinsulinemia, although in the same study, similar insulin levels stimulated endothelial cells to release NO in vitro. In addition, ET-1 assay in arterialized venous blood does not allow differentiation between the contribution of various vascular beds to changes in circulating ET-1. Moreover, since Cardillo et al,[36] using blockade of ET-1 receptors, found a markedly potentiated dependence of the forearm vascular resistance on endogenous ET-1 during 2-hour local hyperinsulinemia in healthy subjects, it cannot be excluded that the stimulation of local ET-1 release in some vascular regions might have occurred at hyperinsulinemia in the present study despite the overall decrease in plasma ET-1 after 2 hours of the clamp. Keeping in mind the methodological drawbacks of using assays of Uno_x and plasma ET-1 as indices of the endogenous formation of respective mediators, it can be assumed that interindividual differences in ΔUno_x and ΔET-1 reflect analogical differences in NO and ET-1 generation. Accordingly, the positive correlation between ΔUno_x and ΔET-1 in healthy subjects may suggest a coupling of vasodilating and vasoconstricting systems during acute hyperinsulinemia. The coupling not only can result from direct insulin-induced stimulation of NO and ET-1 release (as shown by in vitro studies[1-3]) but may also be partially dependent on the ability of ET-1 to potentiate NO generation via endothelial ET-B receptors.[37] Indeed, the fact that the activity of the ET-B receptors continuously attenuates the vasoconstrictive effects of endogenous ET-1 was shown in healthy humans under basal conditions.[38] An impaired ability of endothelial ET-B receptors to stimulate NO release might occur in EH because recently Cardillo et al[38a] have demonstrated forearm vasodilation on selective blockade of ET-B receptors in EH, in contrast to vasoconstriction in normotensives. This could explain why ΔUno_x and ΔET-1, being positively correlated in the control group, were unrelated in our EH men. An alternative explanation of the relationship between stimulation of the L-arginine–NO pathway and generation of ET-1 at acute euglycemic hyperinsulinemia might be sympathetic activation,[34,39] known to occur already at insulin levels fivefold lower than the levels in the present study.[40] In fact, catecholamines stimulate the formation of both NO (acting on α_2- and β-adrenergic endothelial receptors)[41,42] and ET-1,[43] catecholamine-induced NO production is enhanced at insulin concentrations much lower than necessary to promote NO release directly,[3,42,44] and plasma NO_x was shown to positively correlate with muscle nerve sympathetic activity in healthy men.[45] Therefore, it can be concluded that at least several mechanisms could contribute to the observed positive correlation between ΔUno_x and ΔET-1 in normotensives during acute hyperinsulinemia, and further studies of the interaction between these two systems are required.

Uno_x did not increase significantly during the clamp, which is contradictory to results obtained in children by Tsukahara et al,[46] who found an increase in Uno_x of about 100% during acute hyperinsulinemia induced by a bolus dose of insulin 0.1 IU/kg body weight injected intravenously. Nevertheless, in their study, glycemia was not kept constant and peak insulin levels were as high as approximately 7 nmol/L. The proposition that insulin levels higher than those used in the present study are required to increase NOx levels was also suggested by the results of Fragasso et al,[47] who observed marked increases in plasma NOx in response to the same dosage of intravenous insulin as that used by Tsukahara et al,[46] whereas Piatti et al[48] found only slight increases of circulating NOx at euglycemic hyperinsulinemia averaging 230 pmol/L. Although systemic hyperinsulinemia is known to stimulate NO release considerably within the limb circulation even at an insulin level of about 450 pmol/L,[9] ie, lower than in our study, it is noteworthy that the estimation of Uno_x provides only an approximate measure of total NO formation, and the distribution volume of NOx is as large as 28% of the body weight.[23] Accordingly, in the present study, local increases in NO generation could be insufficient to affect Uno_x. Finally, the presence of hyperinsulinemia-induced increases in NO release within skeletal muscle[8,9] does not exclude opposite effects in other vascular beds.

The lack of relationship between ΔUno_x and indices of insulin metabolic sensitivity is in contrast to the report by Higashi et al,[49] who observed a negative correlation between the magnitude of IR and increases in plasma cGMP with L-arginine

infusion in EH. Since this stimulus also produced increases in plasma insulin to about 450 pmol/L and the vascular effects of a similar dose of L-arginine are largely attributable to associated hyperinsulinemia,[50] the report by Higashi et al[49] argues in favor of the previously proposed hypothesis of the coupling of glucose metabolism and NO-dependent dilation in skeletal muscle under hyperinsulinemic conditions.[14] However, our data are consistent with the concept of the dissociation of insulin's ability to stimulate glucose uptake and insulin's effects on local blood flow (mediated via NO release as mentioned previously[8,9]) within skeletal muscle. In agreement with this concept, insulin-stimulated blood flow and insulin-mediated glucose uptake were unrelated when analyzed individually by positron emission tomography in identical regions of interest in femoral muscles of healthy subjects.[51] In addition, Laine et al[52] have recently shown that in obese subjects, administration of bradykinin, a stimulus for NO release, did not attenuate IR as assessed during simultaneous infusion of insulin. Moreover, in a similar population, troglitazone, an insulin sensitizer, did not correct blunted insulin-dependent vasodilation and produced no increase in the NO-dependent fraction of the forearm blood flow in hyperinsulinemic conditions.[53] Finally, insulin sensitivity did not correlate with the magnitude of forearm vasodilation during a hyperinsulinemic-euglycemic clamp in healthy subjects,[16] being only weakly related to this parameter in men with untreated EH.[17]

The described lack of a relationship between insulin sensitivity and the activity of the L-arginine–NO pathway might occur also in normoinsulinemic conditions, contrary to the suggestions of Petrie et al.[18] Indeed, Utriainen et al[19] and Avogaro et al[20] have found no correlation between insulin metabolic sensitivity and the basal forearm response to NO-pathway blockade in normal men[19] and basal forearm NOx balance in diabetics,[20] respectively, and in the present study, basal Uno_x and either the GDR or ISI were not interrelated. Additionally, we have not observed the previously reported[7] negative correlation between the ISI and baseline ET-1 levels.

In conclusion, regardless of the underlying mechanism, the lack of correlation between ΔUno_x and ΔET-1 during a hyperinsulinemic-euglycemic clamp in EH may suggest an impairment of systems governing interactions between the L-arginine–NO pathway and ET-1. Since NO and ET-1 play a pivotal role in the continuous regulation of blood pressure[54,55] and hyperinsulinemia is typical of the postprandial state, this may participate in the pathogenesis of EH. In addition, our study provides biochemical evidence that insulin actions on glucose metabolism and on endothelial mediators appear dissociated.

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