Effects of Endothelin-1 and Nitric Oxide on Glucokinase Activity in Isolated Rat Hepatocytes

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To test the hypothesis that endothelin-1 (ET-1) and nitric oxide (NO) influence glucokinase (GK) activity in an opposite manner, we evaluated the effects of ET-1, L-NAME, an inhibitor of NO synthase, and L-arginine, a substrate for NO synthase, on GK activity and glycogen content in isolated rat hepatocytes. Moreover, to understand the receptor involved in the process, the effects of BQ 788, a specific antagonist of ET_B receptor, and PD 142893, an antagonist of ET_A-ET_B receptors, were also evaluated. GK activity, cyclic guanosine monophosphate (cGMP), and glycogen intracellular content were measured on isolated hepatocytes, while glucose levels and NO as NO_2^-/NO_3^- were determined in the medium. High ET-1 levels induced a 20% decrease of NO_2^-/NO_3^- levels and cGMP intracellular content, followed by a 49% reduction of GK activity and a 15% decrease of glycogen. In parallel, a 10% increase of glucose in the medium was observed. In the presence of L-NAME, GK activity and glycogen levels showed analogous decrements as observed with ET-1. Also in this case, a significant decrease of the intracellular content of cGMP was observed. No synergistic effects of ET-1 and L-NAME were observed. L-Arginine was able to counteract the inhibitory effect of ET-1 on cGMP and GK activity. Glycogen content was slightly but not significantly reduced, and under those conditions, a significant decrease of glucose in the medium was observed. When hepatocytes were incubated with ET-1 plus BQ 788 or ET-1 plus PD 142893, GK activity was unchanged. Interestingly, no changes were observed in NO_2^-/NO_3^- levels and the intracellular content of cGMP was not modified when the antagonists of ET-1 receptors were added to the medium. In conclusion, the present study shows that the NO pathway seems to be an important regulator of GK activity and glycogen content through cGMP activity. In addition, ET-1 seems to be not active per se, but its activity seems mediated by a simultaneous decrease of NO levels.

Insulin-resistant states, particularly non–insulin-dependent diabetes mellitus (NIDDM), are characterized by a failure of insulin to fully suppress glucose production by the liver.[1] The alterations of insulin action at the hepatic level in NIDDM have been related both to impaired glucokinase (GK) activity[2] and to a defect in glycogen storage.[3] In particular, GK plays a key role in the regulation of glucose homeostasis by catalyzing the first step of glycolysis in hepatocytes,[4] since a decrease in GK activity has been reported in NIDDM, causing increased hepatic glucose output.[5] This enzyme is believed to play a major role in hepatic glucose metabolism because of its relative specificity for glucose as substrate and its lack of inhibition by glucose-6-phosphate (glucose-6-P).[6,7] In the liver, GK activity is low during fasting and in diabetes mellitus, and increases after carbohydrate ingestion or in diabetic animals after insulin treatment.[8,9] In the presence of low GK activity, an increase in hepatic glucose output can be shown, while high GK activity promotes glycogen deposition in the liver.[10] Among the hormones that might influence GK activity, insulin plays a leading role as an inducer, even more so in the presence of glucose. In fact, insulin exerts a potent short- and long-term regulatory role in hepatic GK gene expression, since GK was reduced by 90% in the liver of diabetic rats, reaching 65% of nondiabetic controls.[11] Another major determinant of hepatic glucose uptake and production is glycogen storage, and recently, a decrease in glycogen synthesis has been demonstrated in NIDDM[12] and in non-obese first-degree relatives of patients with NIDDM.[3]

Recent studies have shown that endothelial factors such as endothelin-1 (ET-1) and nitric oxide (NO) might modulate hepatic glucose production. Indeed, it has been demonstrated that ET-1, a peptide with potent vasoconstrictor activity, increases hepatic glucose in isolated rat hepatocytes[13] and in perfused rat liver.[14,15]

In contrast, it has been shown that hepatic NO synthesis inhibits glucose output. In particular, Stadler et al[16] increased NO levels in isolated hepatocytes by a combination of cytokines and showed that glucose output was inhibited by 48%, while suppression of NO production by an analog of L-arginine, N^G-monomethyl-L-arginine (L-NMMA), was able to revert this situation. However, the intracellular pathway followed by both of these endothelial factors has not been completely elucidated.

Therefore, to test the hypothesis that ET-1 and NO influence GK activity in an opposite manner, we evaluated the effects of ET-1, L-NAME, an inhibitor of NO synthase, and L-arginine, a substrate for NO synthesis, on GK activity and glycogen content in isolated rat hepatocytes. Our results show that endothelial factors such as ET-1 and NO modulate GK activity and glycogen in isolated rat hepatocytes.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Italy, Calco, Italy) weighing between 225 and 250 g were kept in the animal house of our Institute in controlled conditions (23° ± 1°C with a 12-hour light/dark cycle). The animals were fed standard rat chow and water ad libitum. They were housed according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.*

Incubation Procedures

During the isolation procedure, rats were anesthetized with fluorethane. Hepatocytes were isolated by recirculating collagenase perifusion according to Seglen's method.[17] In particular, the liver was perfused with Hanks solution (pH 7.4, 37°C) for 10 minutes at a constant rate of

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15 mL/min. Then, collagenase type IV (Sigma, Aldrich, Milano, Italy) was allowed to recirculate for about 15 minutes at a constant rate of 20 mL/min. At the end of the procedure, isolated hepatocytes were filtered through sterile gauze and washed 3 times with cold Hanks solution. We accepted cell preparations yielding 85% to 95% viable cells.

After isolation, hepatocytes were preincubated for 60 minutes in nitrate-free RPMI 1640 medium supplemented with penicillin, streptomycin, pyruvate 2 mmol/L, and glutamine 2 mmol/L, pH 7.4, to allow hepatocytes to recover after the isolation procedure before performing metabolic studies.[18] Thereafter, hepatocytes were provided a fresh change of nitrate-free RPMI 1640 medium with penicillin, streptomycin, pyruvate 2 mmol/L, and glutamine 2 mmol/L and supplemented with 12 mmol/L glucose and 1,200 pmol/L insulin (Actrapid HM; Novo Nordisk Farmaceutici, Roma, Italy) and any additional stimuli as indicated. The experiments reported herein were performed with hepatocytes (1×10^6) that were placed in culture for 60 minutes in 35-mm dishes for determination of GK activity, lactate, glucose-6-P, and intracellular cyclic guanosine monophosphate (cGMP) content, while 2×10^6 cells were cultured in 60-mm dishes for 60 minutes to determine intracellular glycogen content. NO_2^-/NO_3^- and glucose levels were measured on the medium.

Cell Culture Conditions

Study 1. In the first part of the study, we evaluated the effects of increased ET-1 levels on hepatic glucose metabolism. The following stimuli were applied for 60 minutes: test G + I, 12 mmol/L glucose and 1,200 pmol/L insulin, considered a control test; test G + I + ET-1, glucose and insulin plus 2×10^{-9} mol/L ET-1 (Neosystem Laboratoire, Strasburg, France), to evaluate the effects of increased ET-1 levels; test G + I + L-NAME, glucose and insulin plus 4×10^{-5} mol/L N^G-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, Milan, Italy), an inhibitor of NO synthase, to understand whether a decrease of NO is able to reproduce the effects of increased ET-1 levels; test G + I + ET-1 + L-NAME, glucose and insulin plus ET-1 and L-NAME, to determine whether a synergistic effect of ET-1 and L-NAME is present; test G + I + L-arginine, glucose and insulin plus 1.72×10^{-3} mol/L L-arginine (Farmaceutici Damor, Napoli, Italy), a precursor of NO synthesis; test G + I + L-arginine + ET-1, glucose and insulin plus ET-1 and L-arginine, to test the hypothesis of whether, by increasing NO levels, it is possible to reverse the effects of increased ET-1 levels. The dose of 2×10^{-9} mol/L ET-1 was chosen after performing a dose-response study that demonstrated a maximum effect on Vmax GK at 2×10^{-9} mol/L ET-1 without any further effect doubling ET-1 levels (Fig 1).

Study 2. In the second part of the study, hepatic receptors involved in the ET-1 effect were studied. Again, stimuli were applied for 60 minutes: test G + I, 12 mmol/L glucose and 1,200 pmol/L insulin as a control test; test G + I + ET-1, glucose and insulin plus 2×10^{-9} mol/L ET-1; test G + I + ET-1 + BQ 788, glucose and insulin plus ET-1 and 1×10^{-5} mol/L BQ 788 (specific antagonist of ET_B receptor; Neosystem Laboratoire, Strasburg, France); and test G + I + ET-1 + PD 142893, glucose and insulin plus ET-1 and 1×10^{-5} mol/L PD 142893 (antagonist of ET_A-ET_B receptors; Neosystem Laboraoire).

Assays

For the determination of GK activity and glucose-6-P, samples were homogenized in 600 µL solution buffer containing 1 mmol/L EDTA, 110 mmol/L KCl, 20 mmol/L K_2HPO_4, and 5 mmol/L dithiothreitol at 4°C and pH 7.7 and centrifuged for 20 minutes at 4°C. Supernatants were then used for the determination of GK activity and glucose-6-P with spectrofluorimetric methods as previously reported by Purrello et al[19] and adapted for use on an automated centrifugal analyzer (COBAS FARA II; Roche, Basel, Switzerland). In particular, for GK, enzymatic activities were measured at different glucose concentrations, 0.06, 0.12, 0.25, and 0.5 mmol/L and 5, 7.5, 10, 15, 20, 25, 50, 80, and 100 mmol, and Vmax values were calculated in each experiment using the Eadie-Hofstee plot. A standard curve was obtained by incubating with the assay reagents 0.3 to 1.0 mol/L glucose-6-P and 1 nmol/L NADH. Enzymes were purchased from Boehringer (Mannheim, Germany). GK activity was normalized by the intracellular protein content. The intraassay coefficient of variation (CV) was 8.0% and interassay CV 8.2%. For the determination of intracellular lactate, samples were pretreated with 3% perchloric acid and measured with a spectrofluorimetric method adapted to an automated centrifugal analyzer (COBAS FARA II) as previously reported.[20]

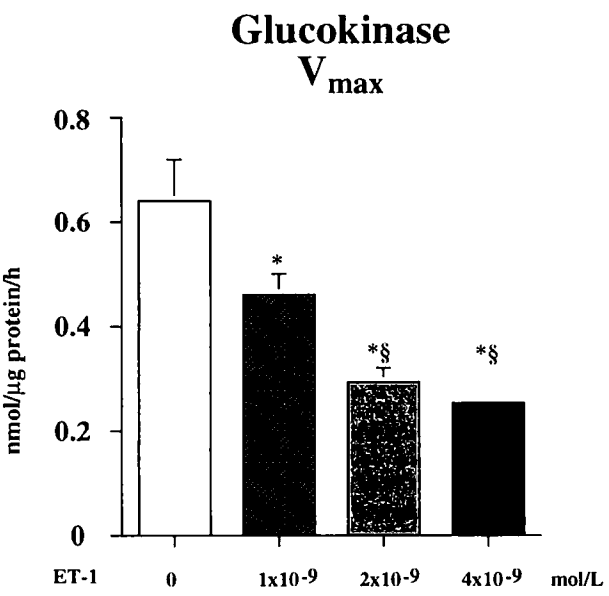

Fig 1. Vmax for GK after a dose-response curve: 12 mmol/L glucose and 1,200 pmol/L insulin in the absence of ET-1 (ET-1 0) or in the presence of ET-1 1×10^{-9} mol/L, or 2×10^{-9} mol/L, or 4×10^{-9} mol/L. Results are the mean ± SEM of 6 similar experiments in duplicate. *$P < .0001$ *v* ET-1 0, § $P < .0001$ *v* ET-1 1×10^{-9} mol/L.

The protein assay was performed on the COBAS FARA II using a commercial kit (BCA Protein; Pierce, Rockford, IL).

Intracellular content of cGMP was measured using a commercial radioimmunoassay kit (Amersham Life Science; Buckinghamshire, England). In particular, at the end of the incubation period, the medium was removed from the cells and 300 µL absolute ethanol was added to permeabilize the cells. The cells were then centrifuged at 4°C for 5 minutes and the supernatants were kept at −80°C until the assay.

Glycogen was assayed in dry tissue after amyloglucosidase digestion. The intraassay CV was 8.7% and interassay CV 11.2%.

Glucose levels in the medium were determined using a commercial enzymatic kit (Roche, Basel, Swizerland). The intraassay CV was 2.0% and interassay CV 2.4%.

NO in the different media was evaluated at the end of the incubation time (60 minutes) by measurement of the end products of their metabolism, ie, nitrite and nitrate (NO_2^-/NO_3^-) levels, using enzymatic catalysis coupled with the Griess reaction as previously reported.[21] Moreover, NO_2^-/NO_3^- levels were also measured at time 0 and the result was subtracted from those obtained at 60 minutes. The intraassay CV was 4.6% and interassay CV 10%.

Statistical Analysis

All data are expressed as the mean ± SEM of 6 experiments assayed in duplicate for each test. ANOVA followed by Fisher's least-squares test were used for evaluation of the data. A P level less than .05 was

considered statistically significant. ΔGlucose, Δlactate, and Δglucose-6-P data are reported as the increment or decrement of glucose, lactate, and glucose-6-P levels in the medium at the end of the 60-minute incubation period during all stimulation tests with respect to test G + I.

RESULTS

Study 1

Figure 2 shows the Vmax for GK, intracellular glycogen content, NO_2^-/NO_3^-, and cGMP levels, and Fig 3 shows Δglucose, Δglucose-6-P, and Δlactate during all stimulation tests.

Glucose and insulin effects (test G + I). At the end of the first incubation period, when hepatocytes were incubated without insulin and glucose, GK activity was 0.29 ± 0.03 nmol/μg protein/h and glycogen levels were 51.6 ± 1.6 mg/g tissue (data not shown). In the presence of glucose and insulin concentrations similar to those found in NIDDM patients during the postprandial period (ie, glucose 12 mmol/L and insulin 1,200 pmol/L, respectively), GK activity increased by 93% ($P < .05$) while glycogen levels were similar versus the absence of insulin and glucose. Under these metabolic conditions, $NO_2^-NO_3^-$ levels were 256.87 ± 17.19 nmol/L; intracellular content of cGMP was 1.47 ± 0.04 pmol/10^{-6} cells.

Effects of high ET-1 levels (test G + I + ET-1). High ET-1 levels determined a 49% reduction of GK activity ($P < .01$ *v* G + I) and a 15% decrease of glycogen content ($P < .05$ *v* G + I). Interestingly, NO_2^-/NO_3^- levels decreased by 20% in the presence of increased ET-1 levels ($P < .01$ *v* G + I), accompanied by a similar decrease of intracellular cGMP levels ($P < .05$ *v* G + I). This determined a 10% increase of glucose levels in the medium (1.19 ± 0.1 mmol/L).

Effects of decreased NO levels (test G + I + L-NAME). When L-NAME, an inhibitor of NO synthase, was administered at a dosage able to induce a similar reduction of NO_2^-/NO_3^- levels as during test G + I + ET-1, GK activity and glycogen levels showed analogous decrements as observed with ET-1 ($P < .01$ *v* G + I and NS *v* G + I + ET-1). Also in this condition, glucose levels increased in the medium (1.47 ± 0.2

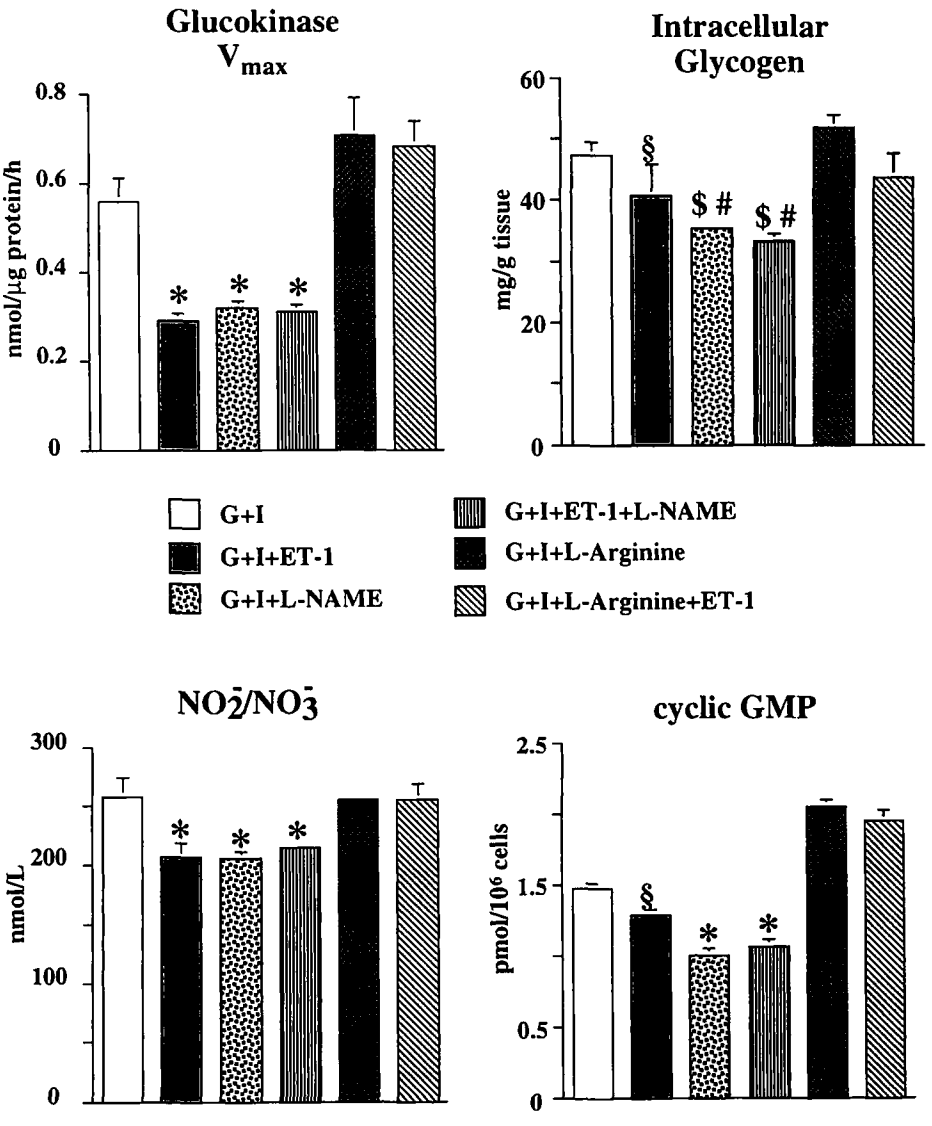

Fig 2. Vmax for GK, NO_2^-/NO_3^- levels, intracellular GMP, and glycogen content during G + I, G + I + ET-1, G + I + L-NAME, G + I + ET-1 + L-NAME, G + I + L-arginine, and G + I + L-arginine + ET-1. Results are the mean ± SEM of 6 similar experiments in duplicate. *$P < .01$ *v* G + I, G + I + L-arginine, and G + I + L-arginine + ET-1; § $P < .05$ *v* G + I.

mmol/L; NS *v* G + I + ET-1), accompanied by a significantly decreased intracellular content of cGMP ($P < .01$ *v* G + I and NS *v* G + I + ET-1).

Synergistic effects of ET-1 and L-NAME (test G + I + ET-1 + L-NAME). To verify whether ET-1 and L-NAME could have synergistic effects on GK activity, ET-1 and L-NAME were added together in the medium. Analogous decrements in NO_2^-/NO_3^- levels ($P < .01$ *v* G + I and NS *v* G + I + ET-1 and G + I + L-NAME) and in the intracellular content of cGMP ($P < .01$ *v* G + I and NS *v* G + I + ET-1 and G + I + L-NAME) were observed. Under these conditions, GK activity and glycogen levels were reduced by about 45% ($P < .01$ *v* G + I and NS *v* G + I + ET-1 and G + I + L-NAME) and about 30% ($P < .01$ *v* G + I and NS *v* G + I + ET-1 and G + I + L-NAME), respectively. This was accompanied by an increase of glucose levels in the medium (NS *v* G + I + ET-1 and G + I + L-NAME). Taking all of these data together, it seems possible to hypothesize the absence of synergistic effects of ET-1 and L-NAME. With ET-1 and L-NAME as a single or combined stimulation, the intracellular content of glucose-6-P similarly decreased, while a significant increase of Δlactate was demonstrated (Fig 3).

Effects of high NO levels (test G + I + L-arginine and test G + I + L-arginine + ET-1). When L-arginine, a precursor of NO synthesis, was added to obtain NO_2^-/NO_3^- levels similar to those of test G + I, GK activity slightly increased (0.71 ± 0.12 *v* 0.56 ± 0.05 nmol/μg protein/h during G + I, NS; $P < .01$ *v* G + I + ET-1, G + I + L-NAME, and G + I + ET-1 + L-NAME). Intracellular glycogen content was similar (51.8 ± 2.0 *v* 47.3 ± 2.2 mg/g tissue, NS; $P < .01$ *v* G + I + ET-1, G + I + L-NAME, and G + I + ET-1 + L-NAME). cGMP levels increased by 28% compared with test G + I (NS). This was accompanied by a significant decrement of glucose levels in the medium ($P < .01$ *v* G + I + ET-1, G + I + L-NAME, and G + I + ET-1 + L-NAME). When ET-1 was added together with G + I + L-arginine, ET-1 was unable to decrease GK activity (NS *v* G + I), while glycogen content was slightly but not significantly reduced (NS *v* G + I). cGMP and Δglucose levels remained stable compared with test G + I + L-arginine ($P < .01$ *v* G + I + ET-1, G + I + L-NAME, and G + I + ET-1 + L-NAME). In the presence of L-arginine with or without ET-1, glucose-6-P and lactate levels were similar to those found in the G + I test (Fig 3).

Study 2

Hepatic receptors involved in ET-1 effects. When hepatocytes were incubated with ET-1 plus BQ 788 (a specific antagonist of ET_B receptor) or ET-1 plus PD 142893 (an antagonist of ET_A-ET_B receptors), GK activity remained stable. Interestingly, similar results were found for NO_2^-/NO_3^- levels, and the intracellular content of cGMP was not modified when the antagonists of ET-1 receptors were added in the medium (Fig 4).

DISCUSSION

Our study demonstrates a novel pathway of ET-1 action in isolated rat hepatocytes: ET-1 decreased GK activity and reduced glycogen content, with a final effect to stimulate glucose release in the medium. This result might contribute to the understanding of the excessive endogenous glucose production that characterizes fasting hyperglycemia in NIDDM patients,[22-24] since elevated ET-1 levels have been found in NIDDM[25-27] and it has been previously demonstrated that ET-1 is capable of producing direct sinusoidal constriction in the intact liver.[28]

The increase in glucose release induced by ET-1 could be determined either by a stimulation of glycogenolysis or by an enhanced gluconeogenesis or both. Previous studies have shown that ET-1 is a potent stimulus of hepatic glucose production, mainly through increased glycogenolysis, both in perfused rat liver[14,15] and in isolated hepatocytes.[13] In particular, both Roden et al[14] and Tran-Thi et al[15] were able to find a maximum effect of ET-1 in the nanomolar range, which is the level used in the present study. Moreover, Chi-Chang et al[29] demonstrated that ET-1 administered intraperitoneally induces insulin resistance in conscious rats without significant hemodynamic changes. The possibility of measuring glucose-6-P and lactate concentrations in the present study allowed us to better understand the metabolic pathways influenced by ET-1. Our data provide support for a critical link between glucose-6-P levels and activation of glycogen synthesis.[30] In fact, we found a positive and significant correlation between glucose-6-P and glycogen concentrations ($r = .31$; $P < .04$), while the presence of ET-1 and L-NAME in the medium determined a significant decrement in glucose-6-P concentrations (Fig 3). In addition, glucose-6-P concentrations were also correlated with GK activity ($r = .41$, $P < .03$). It is well known that GK activity is not inhibited by physiological concentrations of glucose-6-P, the product of the reaction,[31] and in the presence of high glucose levels, the increase of glucose-6-P enhances glycogen synthesis.[30] Taking all of these data together, it is possible to postulate that ET-1 negatively influences glycogen synthase, stimulating glycogenolysis.

To indirectly evaluate the effect of ET-1 on gluconeogenesis, we measured lactate, a validated method to measure glycolytic flux.[32] The presence of increased lactate concentrations strongly suggests that glycolysis and not gluconeogenesis was induced by ET-1 and L-NAME. Since lactate release in the medium significantly increased while glucose-6-P markedly decreased in the presence of ET-1 and L-NAME, one might postulate that the contribution of gluconeogenesis to increased glucose production was not significant under our metabolic conditions.

An element of novelty of the present study is the finding that ET-1 action seems mediated by decreased NO levels, which in turn decrease GK activity through reduced cGMP levels. In fact, when hepatocytes were cultured with L-NAME, an inhibitor of NO synthase, we observed similar results as in the presence of the ET-1 stimulus, namely a decrease in NO_2^-/NO_3^- levels, cGMP intracellular content, GK activity, glucose-6-P, and glycogen content and a consequent increase of glucose levels in the medium, suggesting the presence of a net glucose release in the medium. Once again, in these circumstances, lactate concentrations increased. In contrast, when we cultured hepatocytes with L-arginine, even in the presence of ET-1, opposite results were obtained, and in particular, we observed decreased glucose levels in the medium, suggesting a net glucose uptake from the medium. Tran-Thi et al[15] previously assumed that NO can affect ET-1 action on liver contraction and glycogenolysis, since

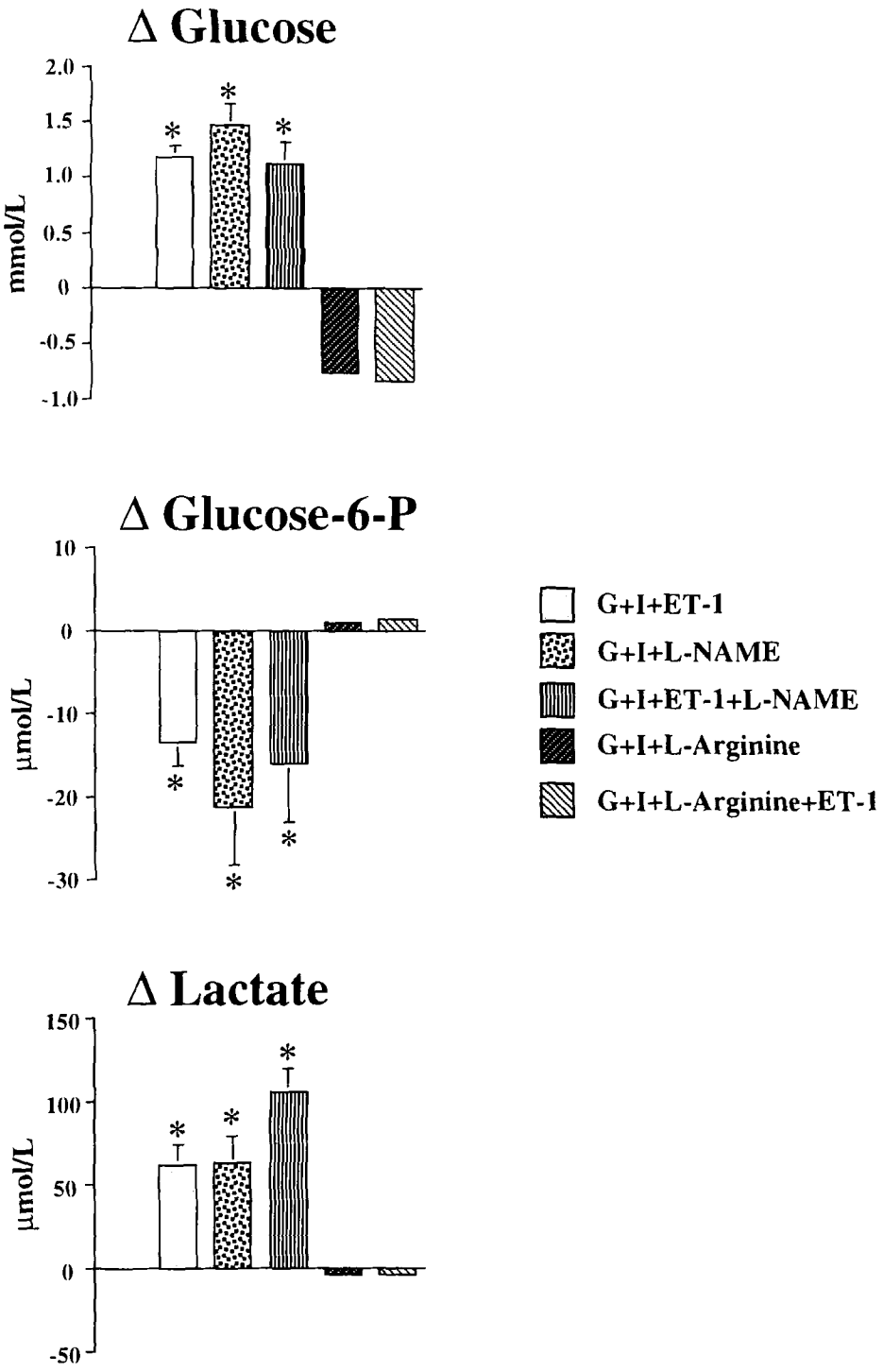

Fig 3. ΔGlucose, Δglucose-6-P, and Δlactate during G + I + ET-1, G + I + L-NAME, G + I + ET-1 + L-NAME, G + I + L-arginine, and G + I + L-arginine + ET-1. Results are the mean ± SEM of 6 similar experiments in duplicate. *$P < .0003$ v G + I + L-arginine and G + I + L-arginine + ET-1.

inhibition of ET-1 action was observed in the perfused liver using a NO donor. The opposite effects of NMA, an inhibitor of NO production, and L-arginine on NO synthesis in hepatocytes have been previously demonstrated by Curran et al.[33] In particular, they were able to demonstrate that NO production was blocked in hepatocytes in the presence of NMA. The inhibitory effect of NMA was overcome when high L-arginine levels (0.24 mmol/L) were added to the medium. The concentration used in the previous study was about 7-fold lower than the concentration of L-arginine used in the present study.

However, it is peculiar that in hepatocytes, ET-1 seems to decrease, not increase, NO and cGMP levels. This is in line with previous data demonstrating that ET-1 caused a dose-dependent inhibition, not an increase, of cGMP production.[34] Moreover, it has been observed that in the pituitary gland, ET-1 significantly inhibited, instead of stimulating, NOS activity, causing a

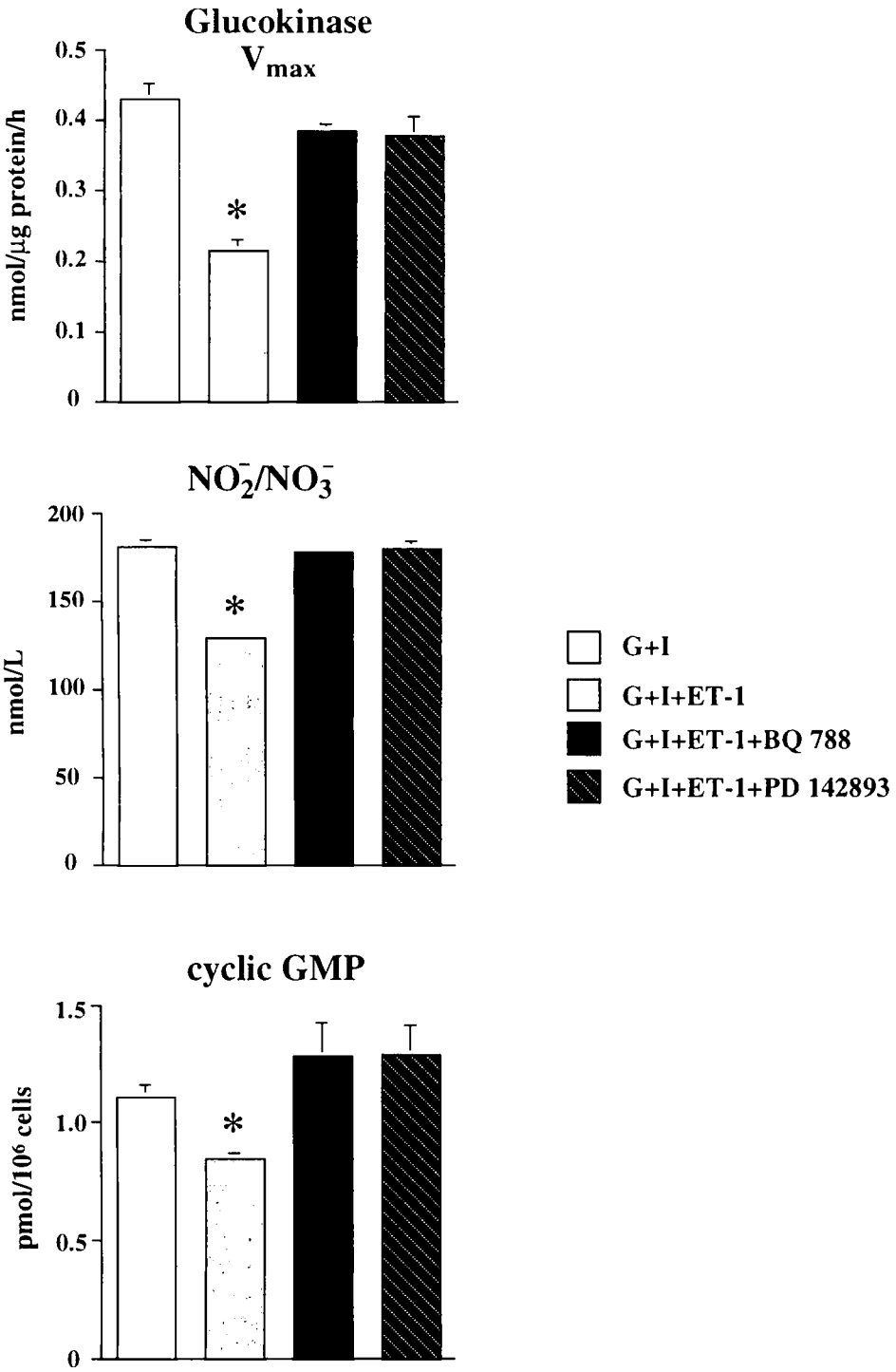

Fig 4. Vmax for GK, NO_2^-/NO_3^- levels, and intracellular GMP content during G + I, G + I + ET-1, G + I + ET-1 + BQ 788, and G + I + ET-1 + PD 142893. Results are the mean ± SEM of 6 similar experiments in duplicate. *$P < .01$ *v* G + I, G + I + ET-1 + BQ 788, and G + I + ET-1 + PD 142893.

reduced prolactin release.[35] On the contrary, it has been shown that ET-1 at levels higher than those used in the present study (10 to 30 nmol/L) increases cGMP levels in glomeruli by stimulating the formation of a NO-like factor that activates soluble guanylate cyclase.[36]

To our knowledge, our results are the first to indicate the pathway followed by ET-1 in inhibiting GK activity. It seems reasonable to postulate that ET-1 acts as an insulin antagonist, since insulin stimulates GK activity after activation of cGMP.[36,37] We do not have data on the ET-1 regulation of GK at the cellular level, ie, whether the effect of ET-1 was related to decreased GK synthesis or to increased activity of the regulatory protein of GK, since we did not perform a study on GK mRNA changes. The fact that GK activity was modified by ET-1, L-NAME, and L-arginine after 60 minutes of incubation seems to support the hypothesis that changes in GK activity were more likely due to

a modulation of enzyme stability through an effect on the regulatory proteins of GK, as already demonstrated in the short-term control.[38] This hypothesis is corroborated also by the fact that even if GK mRNA changes have been observed in 40 minutes, it takes about 4 to 8 hours for the synthesis of new enzyme.[39] However, the evaluation of the ET-1 effect on GK mRNA is an important issue and needs more appropriate investigation.

In addition, from our data, one might postulate that ET-1 decreases NO levels through ET_B receptor in hepatocytes, but our data need further investigations, since the direct evidence of the presence of ET_B receptors in hepatocytes is still lacking, even though there are data showing ET-1 binding to hepatocytes, which progressively increases over 90 minutes, reaching a plateau by 120 minutes.[40] Moreover, it is known that ET_B receptors are expressed in endothelial cells, being the receptors that mediate the release of endothelium-derived relaxing factors/NO and are involved in the stimulation of cGMP.[41-43]

A potential bias of our study is the short time of incubation of the hepatocytes with the different stimuli, as Spence et al[37] observed a sharp increase of cGMP levels 6 hours after the addition of insulin and concomitantly to a second phase of increase in GK activity. However, Nouspikel and Iynedjian[41] found that GK gene transcription was markedly stimulated after 1 hour of insulin stimulation, and Dohi and Murad[44] demonstrated that pyruvate was able to increase cGMP within 2 minutes with a maximal activity at 5 to 15 minutes. Another potential limitation of our study is related to the concentration of ET-1 administered, higher than the levels found even in conditions of increased ET-1 such as diabetes mellitus,[24-26] cardiogenic shock,[45] and endotoxin shock.[46] However, it must be underlined that ET-1 is mostly a paracrine peptide,[47] and presumably, circulating levels do not reflect concentrations in the sinusoids that might be very high during such pathological conditions. Since there are very few data on ET-1 action on glucose metabolism in the liver, further studies are needed to understand the time course of ET-1 activity in modulating hepatic glucose release through its action on cGMP levels and GK activity. This seems particularly interesting, as recent data pointed out the relevance of endothelial factors in inducing insulin resistance. In fact, it has been demonstrated that NO levels, through vasodilation, can affect the disposal of substrates, and in particular glucose metabolism.[48,49]

In addition, Sakamoto et al,[50] studying the Otsuka Long-Evans Tokushima fatty rat, a model of spontaneous NIDDM which develops hyperglycemia, mild obesity, and insulin resistance, showed that the endothelium-dependent vasodilation induced by histamine was decreased, suggesting that endothelial dysfunction may occur even in the early stages of NIDDM. Young et al[51] demonstrated that the NO/cGMP system activates both glucose transport and utilization in isolated skeletal muscle preparations of normal rats. Moreover, a decreased NOS activity was observed in insulin-resistant skeletal muscle of obese rats characterized by reduced glucose utilization.[52] The researchers hypothesized that the NO/cGMP pathway is required for optimal insulin signaling in skeletal muscle, while the presence of a decreased NO/cGMP system would lead to decreased insulin sensitivity. Our data seem to reinforce this hypothesis suggesting that a similar NO/cGMP pathway for optimal insulin signaling is present also in the liver and seems to contribute to a decreased GK activity. This is particularly important since it has been shown that GK activity is severely impaired in NIDDM,[5,53] and the metabolic pathway described in the present study might represent a novel mechanism able to decrease GK activity in NIDDM in the presence of increased ET-1 levels.

In conclusion, the present study shows that the NO pathway seems to be an important regulator of GK activity and glycogen content through cGMP activity in the liver. In addition, ET-1 seems not active per se, but mediated by a simultaneous decrease of NO levels. This represents an additional pathway responsible, at least in part, for the increased glucose production found in insulin-resistant states such as NIDDM.

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