Regulators of hepatic glucose and glycogen metabolism
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CHAPTER 4

The role of nitric oxide in the regulation of glucose kinetics in response to endotoxin in dogs


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Abstract:

The purpose of the present *in vivo* study was to determine the role of nitric oxide (NO) in the regulation of glucose metabolism in response to endotoxin, by blocking NO synthesis with Nω-monomethyl-L-arginine (L-NMMA). In five dogs, the appearance and disappearance rate of glucose (by infusion of [6,6-²H₂]glucose), plasma glucose concentration and plasma hormone concentrations were measured on five different occasions: saline infusion, endotoxin alone (*E.coli*, 1.0 µg/kg, i.v.), and endotoxin administration plus three different doses of primed, continuous infusion of L-NMMA. Endotoxin increased rate of appearance of glucose from 13.7±1.6 to 23.6±3.3 µmol/kg/min (p<0.05), rate of disappearance of glucose from 13.9±1.1 to 24.8±3.1 µmol/kg/min (p<0.001), plasma lactate from 0.5±0.1 to 1.7±0.1 mmol/l (p<0.01), and counterregulatory hormone concentrations. L-NMMA did not affect the rise in rate of appearance and disappearance of glucose, plasma lactate or the counterregulatory hormone response to endotoxin. Plasma glucose levels were not affected by endotoxin, with or without L-NMMA. In conclusion, *in vivo* inhibition of NO synthesis by high dose L-NMMA does not affect glucose metabolism in response to endotoxin, indicating that NO is not a major mediator of glucose metabolism during endotoxemia in dogs.
Introduction

Sepsis is characterized by profound changes in glucose metabolism. In early stages of sepsis, hyperglycemia is found, because of increased glucose production and impaired glucose utilization, both of which are associated with insulin resistance (30). In advanced stages of sepsis, hypoglycemia can be encountered, presumably due to both impaired production and increased utilization of glucose (5,15,25). The pathophysiological mechanisms behind these changes in glucose metabolism during sepsis have not been completely elucidated. Sepsis is also characterized by an increased production of glucose-counterregulatory hormones and cytokines (e.g. interleukin (IL)-1, IL-6 and tumor necrosis factor) (8). When infused in healthy subjects, these hormones and cytokines induce insulin resistance resulting in hyperglycemia (1,33). The increased production of these mediators in sepsis cannot, however, explain hypoglycemia.

Another mediator overproduced in sepsis is nitric oxide (NO) and NO is known to influence glucose metabolism. In vitro studies consistently show that NO is a powerful inhibitor of glucose production by its ability to inhibit gluconeogenesis and glycogenolysis (2,12,20,28,29). In vivo in humans it has been shown that insulin can enhance glucose uptake by inducing vasodilation via direct stimulation of NO production although the quantitative importance of this phenomenon is questioned (3,31). The in vivo data on the potential role of NO in the regulation of glucose production are less clear. In endotoxin-treated rodents inhibition of NO production by either $N^\gamma$-monomethyl-L-arginine (L-NMMA) or knockout of NO synthase (NOS) does not influence glucose production. However, in endotoxin-treated pigs, L-NMMA inhibits glucose production (30). These data suggest that, in certain species, NO is involved in the regulation of glucose metabolism, not only in vitro but also in vivo. Because one of the features of septic shock is overproduction of NO, NO could be an important mediator of hypoglycemia in sepsis via above-mentioned mechanisms.

Endotoxin is often used to mimic the response to inflammation. In endotoxic shock in animals, the increase in NO production is directly related to the degree of hypotension and inhibitors of NOS can reverse or prevent the hypotension induced by endotoxin (19). Via variable hypoperfusion of the organs involved in glucose metabolism, shock in itself will influence glucose metabolism, independently of specific effects of NO. If NO has a specific insulin-like effect on glucose metabolism, it can be expected that blocking NO after endotoxin administration will influence glucose metabolism,
causing higher production and diminished peripheral uptake, without concomitant induction of hemodynamic instability.

NO is a gas with an extremely short half life and has only paracrine effects (19). The involvement of NO in the responses to endotoxin in vivo can only be studied indirectly, e.g. by administration of the L-arginine analog, L-NMMA, a competitive NOS inhibitor (21,23).

To evaluate the role of NO in the in vivo regulation of glucose metabolism after endotoxin, glucose metabolism was studied in five dogs on five occasions: during saline infusion, after endotoxin administration, and after endotoxin administration during three different doses of L-NMMA. In addition, we measured plasma concentrations of L-NMMA. The chosen dose of endotoxin (1.0 µg/kg) results in a major stimulation of glucose production without the induction of hemodynamic instability (18).

Materials and methods

Experimental animals: five male mongrel dogs (weight 35±1kg) were studied on five different occasions. Before the study, all dogs were observed for 2 weeks. Only dogs with normal stools, no febrile disease, normal physical examination and laboratory results (liver function tests, creatinine, leucocyte counts and hemoglobin content) were included. The dogs were fed with a standard diet once a day consisting of 64% carbohydrate, 7% fat, 26% protein, 3% fiber, based on dry weight (D. B. Brok, Hope Farms, Woerden, The Netherlands).

The study was approved by the Ethical Committee for Animal Experiments of the Academic Medical Center, University of Amsterdam and was performed according to the guidelines of the Dutch Law for Animal Experiments.

Study design: Each dog was studied on five different occasions with an interval of at least three weeks between two experiments: saline infusion, endotoxin alone (Escherichia coli, 1.0 µg/kg, i.v.), and after endotoxin administration plus three different doses (on separate occasions) of primed continuous administration of L-NMMA (10 mg/kg, 1 mg/kg/hr (dose 1); 10 mg/kg, 5 mg/kg/hr (dose 2); 30 mg/kg, 5 mg/kg/hr (dose 3). The order in which the studies were performed, was determined by balanced assignment.
The dogs were trained on a daily basis to get acquainted to the experimental setting in order to lie still during the study. Four days before each experiment, a femoral artery catheter was inserted under general anesthesia (Isoflurane 1%. Forene, Abbott Laboratories, Queensbourough, Kent, United Kingdom and N₂O/O₂ [1:1] ventilation). After insertion the catheter was filled with heparin (200 U/ml), closed, and placed in a subcutaneous pocket.

Each study period started at 8.00 AM after an 18-h overnight fast. On the morning of the experiment, the skin around the pocket was anesthetized with lidocaine and opened. Subsequently, the femoral artery catheter was obtained from the pocket and attached to a monitor for continuous intra-arterial blood pressure monitoring (Hewlett Packard) and blood sampling. A catheter was inserted into the right cephalic vein for infusion of saline and ad-ministration of endotoxin. Another catheter was inserted into the left cephalic vein for infusion of [6,6-²H₂]glucose and, depending on the protocol, L-NMMA mono-acetate salt (Calbiochem.-Novabiochem.Co, San Diego, CA, USA). After blood samples were obtained for determination of basal glucose concentrations and enrichments, a primed (17.6 µmol/kg), continuous infusion (0.22 µmol/kg/hr) of [6,6-²H₂]glucose was started. Equilibration of stable isotope enrichment was reached after 2 hours of isotope infusion. At time (t)=0, baseline blood samples were obtained, followed by the administration of endotoxin. In the L-NMMA studies a primed, continuous infusion of L-NMMA was started 5 min prior to endotoxin administration. Endotoxin was given at a dose of 1.0 µg/kg (derived from E. coli 0111:B4, Lot 31H4000, phenol extracted: Sigma Chemical Co., St. Louis, MO, USA) suspended in sterile pyrogen free saline. A stock solution of 100 µg/ml was made, divided into several tubes (Costar, Cambridge, Mass, USA) and stored at -20°C. Prior to injection, the endotoxin solution was thawed at 37°C, vortexed for 3 min, diluted and vortexed again for 10 min.

Measurements and blood samples: rectal body temperature was measured before and every 30 min after endotoxin administration. Mean arterial blood pressure and heart rate were monitored continuously and recorded every 5 min. Arterial blood samples for the determination of plasma glucose concentrations and enrichments were obtained before (t= -120, -15, -10, -5 and 0 min) and after (t= 30, 60, 90, 120, 180 and 240 min) endotoxin administration. Arterial blood samples for the determination of plasma IL-6, lactate, glucagon, insulin, catecholamines, adrenocorticotropic hormone (ACTH), cortisol and L-NMMA levels were obtained before and after (t= 60, 120 180
and 240 min) endotoxin administration. An additional blood sample for the
determination of plasma L-NMMA levels was obtained at t=15 min.

At the end of each experiment all catheters were removed and the dogs remained
under special care for the next 48 hours.

Sample processing: blood samples for determination of plasma glucose concentration
and enrichment. IL-6 and insulin levels were collected in prechilled heparinized tubes
and stored on ice. Blood samples for the measurement of plasma ACTH levels and
hematologic parameters were collected in EDTA tubes. Whole blood was added to
reduced glutathion-EGTA buffer and trasylool for the determination of catecholamines
and glucagon. Perchloracetic acid (10%) was added to blood collected in sodium
fluoride tubes for measurement of lactate. Within 5 min of sampling, blood samples
were centrifuged (3000 rpm. 4°C. 10 min) and plasma was stored at -20°C until
measurement.

Biochemical analysis: all measurements were performed in duplicate. All samples of
each animal were analyzed in the same run. Plasma insulin concentration was
determined by RIA (Insulin RIA 100, Pharmacia Diagnostic AB, Uppsala, Sweden;
intra-assay coefficient of variation 3-5%; inter-assay coefficient of variation 6-9%;
detection limit 2 mU/l), plasma glucagon concentration by RIA (Daiichi Radioisotope
Labs, Tokyo, Japan; intra-assay coefficient of variation 3-5%; inter-assay coefficient
of variation 9-13%; detection limit 15 ng/l), plasma concentrations of norepinephrine
and epinephrine by high performance liquid chromatography and fluorescence
detection, using α-methylnorepinephrine as an internal standard (32) (intra-assay
coefficients of variation for norepinephrine and epinephrine were 6% and 7%, both
for concentrations of 0.5 nmol/l; the inter-assay coefficients of variations were 12%
for concentrations of 1.4 nmol/l for norepinephrine and 14% for concentrations of 0.4
nmol/l for epinephrine); plasma cortisol levels by fluorescence polarisation
immunoassay on technical device X (Abbott Labs, Chicago, IL, USA); intra-assay
coefficient of variation 6.4 and 3.6% at plasma levels of 200 nmol/l and 800 nmol/l,
resp.; interassay coefficient of variation 9.0 and 4.7%, resp.; detection limit was 50
nmol/l). Plasma ACTH levels were determined by ILMA (Nichols Institute, San Juan
Capistrano, CA, USA) with intra-assay coefficient of variation of 3.7% and 4.3% at
plasma levels of respectively 32 ng/ml and 319 ng/ml and interassay coefficient of
variation of 5.1% and 5.4%, respectively.

IL-6 bioactivity was measured with a IL-6 dependent B-9 hybridoma cell line (kindly
provided by L.A. Aarden. Sanguin Blood Supply Foundation. CLB-division,
Amsterdam, The Netherlands) (11). The detection limit was 1 pg/ml. IL-6 standard contained human recombinant IL-6 (kindly provided by L.A. Aarden).

Glucose concentrations and enrichments were determined by Gas Chromatography-Mass Spectrometry (Gas Chromatograph model 5890 II, Mass Spectrometer model 5989 A, Hewlett Packard, Fullerton, CA, USA; column Heliflex AT-1, 30 cm x 0.25 mm x 0.2 μm, Alltech, Deerfield, IL, USA). β-Phenyl-glucose was used as internal standard (20) [intra-assay coefficient of variation 1 to 4%, interassay coefficient of variation 1.5 to 5%; detection limit 1.5 mM (0.5% enriched)]. Plasma L-NMMA concentration was determined by ninhydrin detection on a cation exchanger (Beckman 7300, Beckman Instruments Inc., Mijdrecht, The Netherlands), plasma lactate by enzymatic method (Boehringer Mannheim, Almere, The Netherlands) on a Cobas Bio Centrifugal Analyzer.

Statistical analysis and calculations: all values are expressed as mean±SEM. Non-steady state equations and, when appropriate, (basal values) steady-state equations, were used to calculate the rate of appearance and disappearance (Rq) of glucose as adapted for the use of stable isotopes (26). The distribution volume of glucose was assumed to be 165 ml/kg. Changes from basal values within each study and comparisons between each study at each time point were tested by analysis of variance for randomized block design, using the Newman-Keuls test when appropriate. A P value <0.05 was considered to be statistically significant.

Results

Clinical parameters
Endotoxin administration increased body temperature from 38.2±0.1 to 40.2±0.2 °C (p<0.05), heart rate from 72±10 to 94±5 bpm (p<0.05) and blood pressure from 103±3 to 122±6 mmHg (p<0.05). L-NMMA administration did not affect endotoxin-induced fever or tachycardia (not significant vs. endotoxin). The endotoxin-induced increase in mean blood pressure was highest during the highest dose of L-NMMA infusion (134±6 mmHg vs. endotoxin alone 122±6 mmHg, p<0.05). With the other two lower doses of L-NMMA mean blood pressures were in between those values.

L-NMMA
Before L-NMMA administration, L-NMMA was not detectable in plasma. During L-NMMA infusion, plasma L-NMMA levels were stable and the following values were obtained: 21±3 μmol/l (prime 10 mg/kg, continuous infusion 1 mg/kg/h; p<0.01
versus basal), 53±6 μmol/l (prime 10 mg/kg, continuous infusion 5 mg/kg/h; p<0.01 versus basal), 108±12 μmol/l (prime 30 mg/kg, continuous infusion 5 mg/kg/h; p<0.01 versus basal). These values were significantly different between the three studies (p<0.05) (Fig. 1).

![Graph showing plasma levels of L-NMMA](image)

**Fig 1.** Plasma *N*⁶-monomethyl-L-arginine (L-NMMA) before and after administration of L-NMMA. P(10) C(1) = L-NMMA: prime 10 mg/kg (continuous infusion 1 mg/kg/hr); P(10) C(5) = prime 10 mg/kg (continuous infusion 5 mg/kg/hr); P(30) C(5) = prime 30 mg/kg (continuous infusion 5 mg/kg/hr). Values are means ± SE.

**Glucose metabolism**

Endotoxin increased endogenous glucose production from 13.7±1.6 to 23.6±3.3 μmol/kg/min (p<0.05). L-NMMA, irrespective of the dose, did not affect the rise in glucose production caused by endotoxin. There was no effect of endotoxin on plasma glucose levels with or without L-NMMA. LPS induced a significant increase in the R\text{d} of glucose (p< 0.001). L-NMMA did not influence the LPS induced increase in R\text{d} glucose. Endotoxin increased plasma lactate levels from 0.5±0.1 to 1.7±0.1 mmol/l (p<0.01). The combined administration of endotoxin and L-NMMA resulted in a similar increase in plasma lactate levels (Figs. 2 and 3).
Fig 2. *Plasma lactate, plasma glucose concentration, and glucose production* before and after administration of saline, endotoxin and combined endotoxin and L-NMMA administration. Endotoxin was given at time = 0 as a bolus (1.0 μg/kg). L-NMMA was started 5 min before endotoxin. P<0.05 between endotoxin and saline for plasma lactate and rate of appearance (Ra) of glucose. Values are means ± SE.
Fig 3. Rate of disappearance ($R_d$) of glucose after administration of saline, endotoxin and combined endotoxin and L-NMMA administration. LPS versus saline: $p < 0.001$.

Responses of the counterregulatory hormones and IL-6.

Endotoxin increased plasma insulin levels from 6±1 to 10±2 mU/l ($p<0.05$), glucagon from 29±7 to 172±75 ng/l ($p<0.05$), ACTH from 15±1 to 484±125 ng/l ($p<0.01$) and cortisol from 60±18 to 712±82 nmol/l ($p<0.01$). The effect on insulin secretion was transient, while it was sustained on the other hormones. Endotoxin did not affect plasma catecholamine levels. L-NMMA, irrespective of the dose, did not affect this counterregulatory response to endotoxin. Endotoxin increased plasma IL-6 levels from below detection limit to 47±11 ng/ml ($p<0.001$), and this was not affected by L-NMMA infusion, irrespective of the dose (Figs. 4-6).
Fig 4. Plasma adrenocorticotropic hormone (ACTH) and cortisol concentrations before and after administration of saline, endotoxin and combined endotoxin and L-NMMA administration. Values are means ± SE.
Fig 5. Plasma levels of insulin, glucagon, epinephrine, and norepinephrine before after administration of saline, endotoxin, and combined endotoxin and L-NMMA administration. Values are means ± SE.
Fig 6. Arterial interleukin-6 (IL-6) levels before after administration of saline, endotoxin and combined endotoxin and L-NMMA administration. Values are means ± SE.

Discussion

In this *in vivo* study, the role of NO in the regulation of the glucose production in response to endotoxin was studied in dogs by administration of the NOS inhibitor L-NMMA. Endotoxin increased the glucose production and the $R_d$ of glucose by ~70% with concomitant increases in IL-6 and the counterregulatory hormones, except for the catecholamines. L-NMMA administration increased blood pressure. However, the effect of endotoxin on glucose production, $R_d$ of glucose, glucoregulatory hormones or IL-6 were not affected by escalating doses of L-NMMA infusion. These data indicate, that NO is not a major mediator of the regulation of glucose metabolism after endotoxin administration in dogs.

The question arises whether the present results can be explained by an insufficient inhibition of NO synthesis. Direct measurement of NO production on whole body
level is fraught with major problems. In blood, NO has a half-life of seconds due to its binding to haemoglobin, and changes in plasma NO concentration are therefore often deduced from the concentration of nitrate as a stable end product of NO (19). The plasma nitrate concentration, however, depends not only on NO production but also on nitrate ingestion and nitrate clearance and is therefore not a very sensitive parameter for whole body NO production (6,7,9,14). The only way to evaluate the potential regulatory role of NO on glucose kinetics is by inhibiting NO synthesis concomitantly with indirect measurements of NO activity. L-NMMA, an L-arginine-analog, is a competitive inhibitor of NOS and strongly inhibits NO synthesis in vivo (19). NO is a major regulator of vascular tone by inducing vascular smooth muscle relaxation; intravenous administration of L-NMMA raises blood pressure through an increase in systemic vascular resistance (19). A rise in blood pressure during L-NMMA infusion can therefore be used as a sign of inhibition of NO production. In vitro it has been shown that L-NMMA at concentrations of ~ 40 μM causes 40-50% suppression of LPS induced NO-synthesis and, at concentrations of ~100 μM, a 60-80% suppression of NO synthesis in both macrophages and endothelial cells (16,23). NO concentration in exhaled air reflects endogenous NO production in health and disease, including local production of NO in the respiratory tract and lungs (17). Comparison of in vivo data on the influence of L-NMMA administration on the NO production in exhaled air obtained by our group in humans with those obtained by MacAllister et al. (16) in vitro in macrophages indicates that the same concentration of L-NMMA induces the same degree of suppression of NO production in vitro as in vivo (F. Sprangers, W.T. Jellema, C.E. Lopuhaä, E. Endert, M.T. Ackermans, J.J. van Lieshout, J.S. van der Zee, J.A. Romijn and H.P. Sauerwein, unpublished observations). These in vitro and in vivo data suggest that in our study, NO production, at least in macrophages and endothelial cells, was inhibited for 60-80% with the highest L-NMMA dose. It seems unlikely that insufficiently high L-NMMA concentrations would reach the Kupffer cells. L-NMMA is water soluble and, considering the fenestration of the intrahepatic vessels, hepatocytes and Kupffer cells would also have been exposed to high L-NMMA concentrations. It is therefore unlikely that the present results can be explained by insufficient inhibition of NO synthesis. In vitro, NO inhibits hepatic glucose production, glycogenolysis, and gluconeogenesis (2,12,20,28,29). From these in vitro data, one could hypothesize that NO may have an inhibitory role in the regulation of glucose production after endotoxin. Glucose
production in our dogs increased by 72% to 23.6±3.3 μmol/kg/min after endotoxin. If this would have been the highest possible glucose production rate in postabsorptive dogs, then, with L-NNMA, no further increase could be expected. However, Wolfe et al. (35) have shown that glucose production rate can be increase by another 100% in conscious postabsorptive dogs with a higher dose of endotoxin. Another explanation for the absence of an effect of L-NMMA on endotoxin-induced glucose production could be that the observation period of 4 h was too short to study the role of nitric oxide by blocking its synthesis. This is also unlikely because a near-maximal induction of NOS in hepatocytes has been described to be reached within 2-3 h (4,13,14,27). The most likely explanation for our findings is that NO has no important role in the regulation of glucose production in endotoxia in dogs. This conclusion is supported by the data obtained by Ou et al. (22) in rodents. They infused L-NMMA in the portal vein of rats for 12 hours and reduced the endotoxin-induced increase in plasma nitrate and nitrite by ~ 60%. There were no changes in the suppression induced by endotoxin in gluconeogenesis. In mice they showed that the gluconeogenic response after endotoxin was not different between knock-out mice without inducible NOS and wild-type mice. They concluded that the effect of endotoxia on gluconeogenesis in intact organs and in vivo in rodents is induced via NO-independent pathways. The data by Träger et al. (30) indicate the existence of species differences in the role of NO in the induction of dysregulation of glucose metabolism by LPS. However, their data also suggest that NO stimulates glucose production, a finding in complete contradiction to all other data published in literature about this subject and therefore needing conformation.

The absence of an effect of L-NMMA on the LPS-stimulated Rd of glucose in our study is less convincing than the absence of an effect on glucose production. Although there was no statistical difference between the LPS-stimulated Rd of glucose with and without L-NMMA, the values obtained during L-NMMA seemed to be lower than during LPS alone. This absence of a statistical effect can be due to the large variability of Rd glucose after LPS alone in combination with the small sample size. However, the absence of any dose-effect relationship between L-NMMA dose and Rd glucose makes this possibility less likely.

In conclusion, in vivo inhibition of NO synthesis by high dose L-NMMA in dogs does not affect glucose production and probably does not affect glucose uptake in response to endotoxin, indicating that, in dogs like in rodents, NO is not a major mediator of glucose metabolism during endotoxia.
Chapter 4

Acknowledgements

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References


