The importance of the arginine/ADMA ratio on the bioavailability of nitric oxide and organ function in critically ill patients

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Chapter 5

Modulation of regional nitric oxide metabolism: Blood glucose control or insulin?

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Abstract

Introduction
Tight glycemic control by intensive insulin therapy (IIT) reduces morbidity and mortality of critically ill patients. As potential mechanisms contributing to the clinical benefits we hypothesized that glycemic control affects regional NO-bioavailability by changing NO synthases (NOS) activity, NOS transcription, NOS substrate availability or the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) levels.

Methods
In a rabbit model of prolonged critical illness we assessed the relative impact of maintaining normal insulin/normoglycemia (n=8), high insulin/normoglycemia (n=8), normal insulin/hyperglycemia (n=9) and high insulin/hyperglycemia (n=8) plasma-levels over 7 days on activity and gene expression of endothelial and inducible NOS isoforms in muscle, liver and aorta biopsies and on plasma-levels of NO, arginine and ADMA.

Results
Compared with normoglycemic groups, both hyperglycemic groups revealed 53% higher day 3 NO plasma-levels ($p<0.05$), 40% lower NOS-activity in muscle ($p<0.01$) and 35% lower endothelium-mediated relaxation of aortic rings ($p<0.01$), 515% higher gene expression of iNOS in muscle ($p<0.01$) and 99% higher eNOS gene expression in aorta ($p<0.01$). Only the hyperglycemic/hyperinsulinemic group showed lower arginine plasma-levels (53% lower, $p<0.0001$). Compared to healthy controls, normoglycemic animals revealed 33% lower ADMA-levels ($p<0.05$).

Conclusions
In this animal model of prolonged critical illness, maintaining normoglycemia, and not glycemia independent actions of insulin, prevented excessive systemic NO release on day 3 and appeared to preserve local endothelial function. Factors contributing to this finding may comprise direct endothelial cell damage, direct effects on the enzyme activity, decreased substrate availability or less NO-induced inhibition.

Introduction

As part of the stress response, critically ill patients become hyperglycemic, brought about by both insulin resistance and relative insulin deficiency. The dogma that this "diabetes of injury" is advantageous for survival was challenged by the finding that hyperglycemia is associated with adverse outcome in a number of critical medical conditions (1), whereas evidence from clinical trials suggests that normalising blood glucose by intensive insulin therapy (IIT) reduces morbidity and mortality of critically ill patients (2-4). However, hitherto it remains unclear whether this results from glycemic control or glycemia independent actions of insulin. Among others (5), the modulation of nitric oxide (NO) metabolism emerges as a potential mechanism explaining the clinical benefits of IIT (6-8).

NO is released from the amino acid arginine by a family of enzymes named NO synthase (NOS). Arginine is abundantly available since its plasma-levels markedly exceed the K_m of NOS. The constitutive NOS isoform eNOS is mainly expressed in vascular endothelium, but also in other cell types such as muscle and liver cells (9), and accounts for basal NO production, regulating blood pressure and microcirculatory blood flow (10). eNOS expression and activity can be modulated by the availability of cofactors and various stimuli such as endotoxin, cytokines, or hyperglycemia (9-11). These stimuli can also induce a second NOS isoform, inducible NOS (iNOS) in several tissues (12). iNOS is considered to be a high capacity NO producing enzyme, liberating high amounts of NO that may reduce blood pressure and enlarge the risk of organ failure and death from acute systemic inflammation (10;13).

Inhibitors of NOS reduce the NO production and, consecutively, counteract NO mediated (excessive) vasodilatation. However, administration of unselective NOS inhibitors increased mortality in patients in septic shock despite positively affecting blood pressure (14). Also high plasma-levels of asymmetric dimethylarginine (ADMA), an endogenous, unselective NOS inhibitor, are associated with increased mortality in critical illness (15). ADMA is a derivate of arginine, synthesized when arginine residues in proteins are methylated, and is released from the bodies' protein pool during catabolism.

In critical illness, both overwhelming NO production with ensuing cardiovascular collapse (10;13) and (regional) NO deficiency (14) resulting in impaired microcirculation and organ dysfunction, can occur. For survival, however, it appears crucial to prevent both excessive and impaired NO release. Our group suggested that IIT plays an important role in this context since it prevented excessively elevated NO plasma levels (6). Furthermore, IIT attenuated elevated ADMA levels (8) and iNOS transcription in post mortem muscle and liver tissue samples of ICU patients (6) enrolled in the clinical trial (2).

We here further unravel mechanisms behind the clinical benefits of IIT in tissue biopsies of skeletal muscle, liver and aorta with focus on regional NO

metabolism employing an animal model of prolonged critical illness. We chose these tissues because in post mortem biopsies from patients who died in our ICU, we observed effects of IIT on NOS transcription in muscle and liver (6). Moreover, in prior animal experiments, we observed effects on vascular endothelial function (7).
We hypothesized that either glycemic control or glycemia independent actions of insulin affect NO generation by modulating NOS transcription or posttranscriptional modulation of the enzyme activity. Hence in tissue biopsies we quantified gene expression of eNOS and iNOS and measured GTP cyclohydrolase I (GTPCH) gene expression as a marker of tetrahydrobiopterin (BH4) bioavailability, an important NOS cofactor. Moreover, we measured NOS substrate availability and the levels of the endogenous NOS inhibitor ADMA in plasma.
Part of the data was previously presented at an international meeting and published as abstracts (16;17).

Material and Methods

All animals were treated according to the "Principals of Laboratory Animal Care" formulated by the U.S. National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the U.S. National Institute of Health. The protocol was approved by the local ethical committee for animal research.
Our model, revealing a similar biphasic pattern of endocrine and biochemical changes as what is documented in patients with critical illness, was described in detail elsewhere (7). Briefly, prolonged critical illness was brought about by a third degree burn injury of 20% body surface area in adult, male New Zealand White rabbits under general anaesthesia. Additionally, catheters were placed into the right jugular vein and right carotid artery, respectively, to allow continuous intra venous infusion and repetitive arterial blood sampling. Endogenous insulin deficiency was induced by alloxan monohydrate (150 mg kg^{-1}). Continuous parenteral nutrition (TPN, see electronic supplementary material) assured basal glucose intake and equal physiologic amino acid and fat intake in all groups (Table 1).
We modulated both blood glucose and insulin plasma-levels within a physiological range independently from each other over a period of 7 days post burn injury. Intravenous insulin (Actrapid, Novo Nordisk, Begsvaerd, Denmark) was administered continuously in two fixed doses, low physiologic and high physiologic, respectively. Blood glucose was titrated to target by adjusting the rate of a continuous intravenous glucose infusion (Glucose 50%, Baxter, Lessiness, Belgium) (table 1). The following 4 study groups resulted (Table 1): group 1: normal insulin levels and normoglycemia (NI/NG), group 2: high insulin levels and normoglycemia (HI/NG), group 3: normal insulin levels and

hyperglycemia (NI/HG), and group 4: high insulin levels and hyperglycemia (HI/HG). Animals were randomized to groups by sealed envelopes.

On day 7, animals were sacrificed and tissue samples of skeletal muscle (quadriceps femoris) and liver were taken and immediately snapfrozen in liquid nitrogen. The thoracic aorta was carefully separated from connective tissue, a sample was snapfrozen and 0.5 cm thick rings were placed into modified Krebs solution for measuring endothelial NOS-activity as described elsewhere (7).

Animals without chronic instrumentation, alloxan injection and burn injury served as healthy controls. Blood was taken prior to sacrifice via the central ear artery. At sacrifice, control animals had mean (±SEM) insulin levels of 23.7 (±27) mU/L and glucose levels of 6.7 (±0.6) mmol/L.

Table 1. Workflow of the experiment, doses of the interventions and results of metabolic control.

		Group 1: NI/NG	Group 2: HI/NG	Group 3: NI/HG	Group 4: HI/HG
Time	Intervention	Normal insulin Normoglycemia	High insulin Normoglycemia	Normal insulin Hyperglycemia	High insulin Hyperglycemia
Day -1	Alloxan IV (mg/kg)	150	150	150	150
Day 0	Instrumentation	Anaesthesia, burn injury, placing venous and arterial lines			
	IV Hartmann solution (ml/h)	18	18	18	18
		Start TPN (12 ml h^{-1}) and interventions			
	Insulin dose (U/kg/d)	1.2	4.0	1.2	4.0
	Target blood glucose (mmol/L)	4.4-6.1	4.4-6.0	13.8-19.2	13.8-19.2
	Nutritional intake				
Day 1-7	Glucose intake (g/d) *	27.4 (±13.9)	47.2 (±17.7)	64.3 (±21.9)	75.9 (±23.4)
	Fat intake (g/d)	3.5 (±0.2)	3.3 (±0.2)	3.4 (±0.3)	3.5 (±0.2)
	Amino acid intake (g/d)	3.5 (±0.2)	3.3 (±0.2)	3.4 (±0.3)	3.5 (±0.2)
	Metabolic control				
	Plasma insulin (mU/L) †	43.3 (±16.6)	188 (±47.5)	47.3 (±17.9)	199.8 (±78.1)
	Blood glucose (mmol/L) ‡	5.1 (±1.9)	4.7 (±2.0)	18.0 (±6.7)	17.3 (±6.0)
	Number of survivors day 7	8	8	9	8
Day 7		Anaesthesia, tissue samples, aortic rings			

Hartmann solution (18 ml h^{-1} on day 0) and parenteral nutrition (12 ml h^{-1}; mixture of fat, amino acids and glucose from day 1 to 7) were continuously infused in equal doses in all groups. Insulin was continuously administered at fixed speed from day 0 to day 7. The speed of an infusion pump with glucose 50% (G 50%) was adjusted to regulate glycemia from day 0 to day 7. Data presented as mean±SD over 7 days. TPN: total parenteral nutrition.

(*) Glucose intake differed significantly among groups ($p<0.03$). (†) Plasma insulin levels differed significantly among groups ($p<0.001$), not between both normal insulin groups ($p=0.9$) nor between both high insulin groups ($p=0.5$). High insulin groups were different ($p<0.0001$) from the low insulin groups. (‡) Blood glucose differed significantly among

groups ($p<0.0001$), not between both normoglycemic groups ($p=0.6$), nor between both hyperglycemic groups ($p=0.4$). Hyperglycemic groups were different from normoglycemic groups ($p<0.0001$).

Assays

Blood glucose was quantified in arterial blood sampled from an indwelling line using a blood gas analyzer (ABL analyzer, Radiometer, Copenhagen, Denmark) 3 to 7 times daily to allow tight adjustment of blood glucose. Supplementary, before the intervention (baseline) and on days 3, 5, and 7 post burn injury, respectively, 4 ml blood was collected and plasma was stored at -80°C until analysis. All analyses were conducted blinded to randomisation in duplicate within a single assay run. All chemicals were obtained from Sigma (Bornem, Belgium) unless indicated otherwise. Plasma insulin was measured by RIA using a guinea pig derived antibody as described elsewhere (18).

Circulating levels of NO, arginine and ADMA

NO levels in plasma were quantified by measurement of plasma nitrate plus nitrite levels using nitrate reductase and the colorimetric Griess reaction as previously described elsewhere (19) and in the electronic supplementary material. The concentration of arginine and ADMA were determined by high performance liquid chromatography (HPLC) with fluorescence detection as described previously (20).

NOS-activity

Please find a detailed description in the electronic supplementary material. In tissue samples the conversion of L-^{3}H-arginine to L-^{3}H-citrulline was used as an index of NOS-activity (adapted from (21)). Data were expressed as fmol produced NO per gram protein. We used the relaxation of a norephedrine induced preconstriction by 10 µmol l^{-1} Ach in isolated perfused aortic rings as an indirect measure of NOS-activity in the vessel as described previously (7). Data are expressed as percentage relaxation of a norephedrine induced constriction.

Gene expression

Gene expression was quantified by TaqMan real time PCR. The combinations of primers and probes for eNOS, iNOS and GTP cyclohydrolase I (GTPCH) can be obtained from the electronic supplementary material. Data were corrected for HPRT mRNA content. All real time PCRs were run together with a series of 10 fold dilutions (from 10^{8} to 10^{2} copies) for generation of a standard curve. Individual samples with a copy number coefficient of variation greater than 20% were reanalyzed. Messenger RNA (mRNA) from skeletal muscle, liver and aorta biopsies was isolated with RNAeasy Mini Columns (Qiagen, Venlo, The Netherlands) and quantified (NanoDrop spectrophotometer, Wilmington, DE, USA). Total RNA (muscle 0.6 µg, liver 1 µg, aorta 0.6 µg) was reverse

transcribed and external standards were generated as described previously (6). Reactions lacking reverse transcriptase were run to generate controls for assessment of genomic DNA contamination. cDNA was quantified in real time with the ABI PRISM 7500 sequence detector (Applied Biosystems, Lennik, Belgium). GTPCH is the rate limiting enzyme of Tetrahydrobiopterin BH4 synthesis, an important co factor of NOS; its gene expression mirrors BH4 bioavailability (22). We therefore quantified GTPCH gene expression in the biopsies as described above.

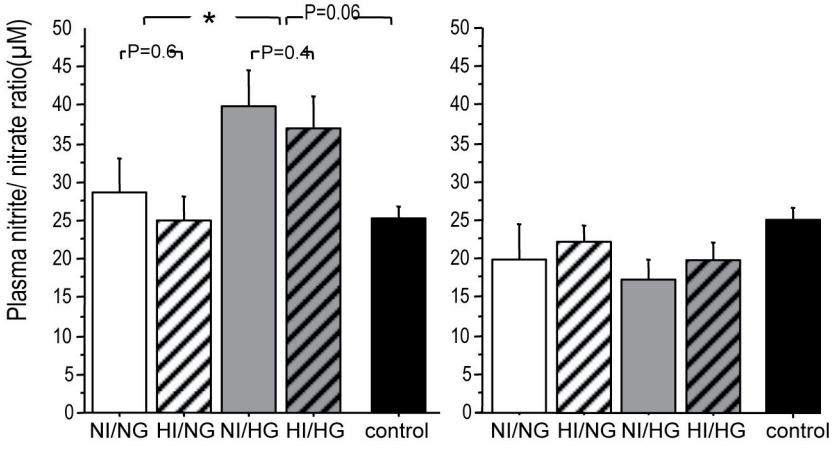
Statistical analysis

Results are expressed as mean ± SEM. Data were analysed by ANOVA for repeated measurements (plasma insulin and blood glucose levels over time), Kruskal-Wallis and Mann-Whitney U tests. Correlation analysis was performed by linear regression (Pearson Coefficient). A two tailed p-value <0.05 was considered significant.

Results

Forty-seven animals were needed to reach designated group size (Number of survivors to day 7: NING:8, HI/NG:8, NI/HG:9, HI/HG:8; healthy controls:8).
Plasma NO levels were elevated in both hyperglycemic groups on day 3 ($p<0.05$ versus normoglycemic groups). In the survivors on day 7, NO levels were not significantly different among tested groups ($p=0.2$, Figure 1).

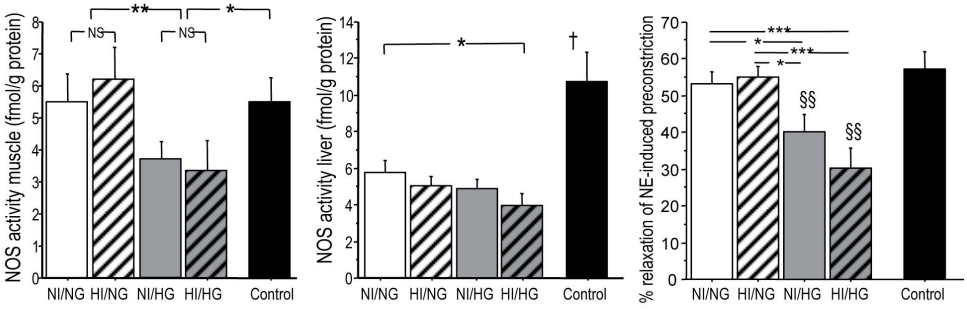
Figure 1. Plasma nitrite/nitrate ratio as a surrogate parameter of plasma NO levels on day 3 (left), and day 7 (right).



*p<0.05.

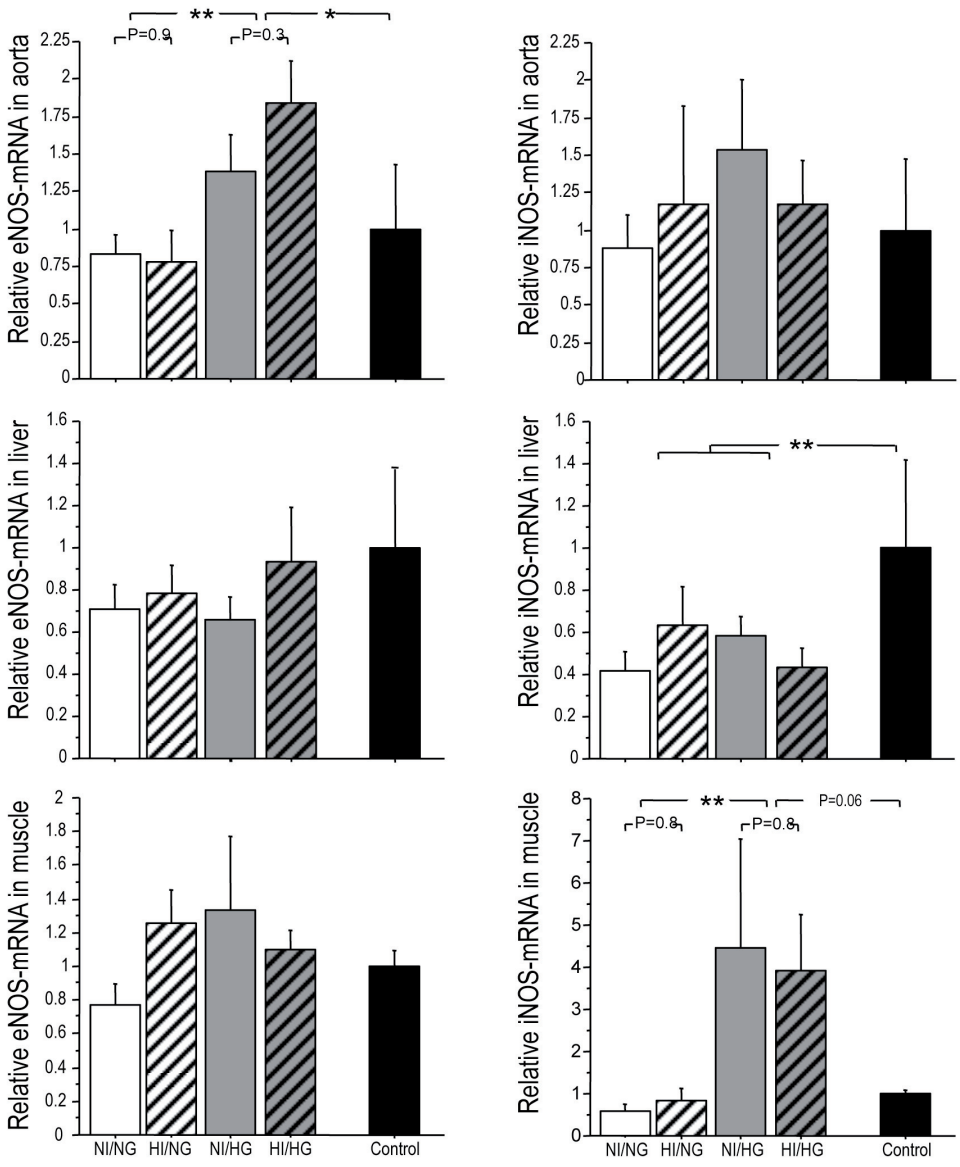
In skeletal muscle biopsies, NOS-activity was lower in hyperglycemic animals compared to normoglycemic and healthy controls ($p<0.01$). In liver biopsies, NOS-activity was lower in all animals compared to healthy controls ($p<0.01$), most pronounced in HI/HG animals (Figure 2). In the aortic rings, endothelial function, as indicated by the percent relaxation in response to Ach of NE-induced preconstriction, was maintained in normoglycemic animals but was diminished in both hyperglycemic groups ($p<0.001$; Figure 2).

Figure 2. NOS activity in muscle, liver and aortic tissue.



Endothelial function expressed as NOS-activity as fmol NO produced per gram protein in 20 minutes incubation in muscle (left) and liver (median) tissue homogenates, and as maximal relaxation of a norephedrine (NE)-induced preconstricion by Ach in aortic tissue (right). †$p<0.001$ vs all groups; *$p<0.05$, **$p<0.01$, ***$p<0.001$, §§$p<0.01$ versus controls.

Figure 3. Relative eNOS (left) and iNOS (right) mRNA expression in aorta (upper panel), liver (middle panel), and skeletal muscle (lower panal) tissue.

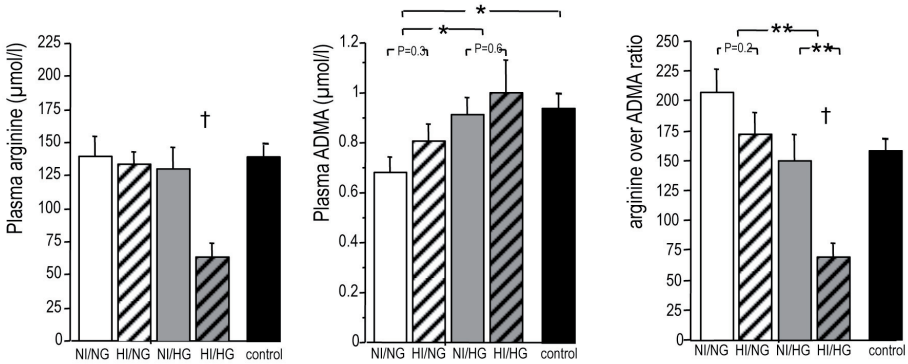


*p<0.05; **p<0.01.

eNOS gene expression in aortic tissue was increased in hyperglycemic animals compared to normoglycemia and healthy controls (p<0.01; Figure 3). There was no significant effect on eNOS expression in muscle or liver (Figure 3). In muscle, iNOS expression was higher in hyperglycemic animals in comparison to

normoglycemic and healthy animals (p<0.01; Figure 3). In liver, iNOS expression in liver was lower in prolonged ill rabbits, compared to healthy animals (p=0.043) without significant differences among groups (Figure 3). In aortic tissue we could not detect any significant effect on iNOS gene transcription. GTPCH gene expression revealed no significant differences among groups and healthy controls in liver, muscle or aorta (data not shown).
ADMA plasma-levels on day 7 were lower in the two normoglycemic groups compared to hyperglycemic animals (p<0.05) and to healthy controls (p<0.05; Figure 4). Plasma arginine levels were lower in HI/HG animals compared to healthy controls (p<0.05; Figure 4). Plasma arginine levels correlated negatively with NOS-activity in muscle (R^2=0.138, p=0.03) and in aorta (R^2=0.226, p=0.007).

Figure 4. Plasma levels of arginine (left), ADMA (middle) and arginine/ADMA ratio (right) on day 7.



*p<0.05; **p<0.01, † p<0.01 vs. all other groups and controls.

Discussion

In our animal model of prolonged critical illness, maintaining normoglycemia, and not glycemia independent actions of insulin, prevented excessive systemic NO release on day 3 and appeared to preserve endothelial function, NOS gene expression and activity in skeletal muscle and aorta biopsies, but not in liver. Hyperglycemic animals showed a rise in NO levels on day 3, elevated NOS gene expression, but decreased endothelial function in muscle and aorta biopsies, not in liver.
Animals of both hyperglycemic groups revealed increased NO plasma-levels on day 3 post burn injury, corroborating previous observations in patients (13). High circulating amounts of NO have been related to hypotension and reduced tissue perfusion and increase the risk of organ failure and death from acute systemic inflammation (10;13). Independent from the insulin levels, in both

normoglycemic groups the rise in circulating NO on day 3 was prevented. Hence, the lowering effect of IIT on plasma NO levels previously observed in critically ill humans (6) is most likely a results from strict glycemic control rather than from glycemia independent effects of insulin. On day 7, however, NO levels no longer differed among groups. This does not contradict the findings in humans, since, in contrast to our study in humans where non survivors were selected, for the final analyses on day 7 we possibly selected those animals with a less severe course of illness since mortality in the hyperglycemic groups surmounted the mortality in normoglycemic groups (7). NO levels were not elevated in normoglycemic animals on the studied days. Possibly, due to necessary blood sampling limitation, we missed an early peak in NO production that may have been affected but not detected in our experiments.

Circulating NO levels do not necessarily reflect regional or local NO availability. Indeed, hyperglycemic animals revealed a pronounced decrease in NOS-activity in skeletal muscle and decreased Ach induced relaxation in aortic tissue despite similar circulating NO levels on day 7. We need to point out that although the decreased relaxation of aortic rings might be induced by decreased NOS-activity, it also may represent direct hyperglycemia-induced damage to the endothelial cells. Since the contraction-force of the rings in response to NE did not differ among groups (data not shown) we can however exclude a bias by an impact of glycemic control on pressor-reactivity. In liver the pattern differed, since NOS-activity decreased in all sick animals compared with healthy controls, most pronounced in HI/HG animals. Our results corroborate findings in various models of diabetes mellitus where hyperglycemia induced reduction of NOS-activity (23). Insulin may have vasodilatatory properties (9;11) and can augment the NO induced cGMP dependent pathway (24). However, like in other models of insulin resistance (11), we could not detect any glycemia independent actions of insulin on NOS-activity. This might be explained by hyperglycemia and systemic inflammation both impairing insulin signalling (25;26). It has to be remarked that data on insulin signalling during critical illness are scarce and that extrapolating data from diabetes mellitus to the setting of the "diabetes of injury" should be done with great caution.

Earlier studies suggest that NOS-activity is mainly regulated at the level of gene transcription (12). Modulation of gene transcription might largely explain our findings in liver since both NOS-activity and iNOS expression were low in all critically ill animals. However, NOS-activity and iNOS expression in muscle and NOS-activity and eNOS expression in aorta responded to hyperglycemia in an opposite way. Our findings reflect the situation in human critical illness where iNOS expression in muscle was increased during hyperglycemia (6). Also in other experiments iNOS induction was seen as a part of systemic inflammation (12;27) with hyperglycemia amplifying the effect in neurons (28), myocardium (29) or cell cultures (30). Maintaining normoglycemia can prevent excessive inflammation thus minimizing iNOS induction via inflammatory cytokines (6;31).

In our model alike in models of diabetes mellitus, insulin did not show any modulating effects on either iNOS or eNOS expression on both levels of glycemia. Again, this might be explained by an inhibition of the PI3K pathway of insulin signalling by e.g. inflammation and hyperglycemia (9;11). Since NOS-activity was suppressed during hyperglycemia despite increased NOS gene transcription, our findings suggest posttranslational regulation of NOS-activity (12) rather than an effect on gene expression as the major determinant of NOS-activity.

Since in our experiments the essential cofactors of NOS, calcium and NADPH, were available in the assays in equal amounts and GTPCH gene transcription was found to be unchanged, largely reflecting similar bioavailability of BH4 (22) in all groups, we consider these factors of minor relevance.

Griscavage and colleagues previously showed that by a negative feedback mechanism, elevated NO levels cause a decrease in NOS-activity. This effect is promoted by a higher oxidation state of the haem iron within the NOS molecule (32). Indeed, on day 3, plasma NO levels were elevated during hyperglycemia and thus probably contributed to the later suppression of NOS-activity. BH4 counteracts this suppression of NOS by NO; although we did not detect any changes in GTPCH expression as a surrogate of equal BH4 availability in any organ (at day 7), we cannot rule out that there might have been an effect in the early course of our experiment.

Apart from possible direct effects of hyperglycemia and NO on NOS-activity, altered NOS substrate kinetics emerges as a reasonable explanation. Indeed, in hyperglycemic animals the plasma concentration of the sole substrate for NOS, arginine, dropped. The arginine levels correlated negatively, albeit weakly, with NOS-activity in muscle and aorta. Lee and colleagues showed that substrate deprivation can reduce NOS-activity (33). The suppressed activity occurred despite maintained intracellular arginine levels surmounting the K_m of NOS, theoretically sufficient to assure NOS saturation (33). In human type II diabetes arginine was shown to be an important regulator of local NOS-activity and thus local NO bioavailability (34).

Since critical illness reflects a complex entity, we cannot exclude other potential posttranslational modifiers of local NO bioavailability, such as oxidative stress.

In diabetes mellitus, elevated ADMA levels occur in the context of insulin resistance (35) and hyperglycemia (36) and render patients at risk for cardiovascular complications (37). Also in human critical illness, ADMA levels correlate with adverse outcome (15) whereas bringing down ADMA levels by IIT has shown to correlate with survival benefits (8). Plasma ADMA levels were however, not elevated in the hyperglycemic groups compared to healthy controls although normoglycemia did induce a decrease in circulating ADMA levels.

Our current animal experiment shows that maintaining normoglycemia rather than insulin is crucial for preserving NOS-activity and expression, physiological arginine and ADMA levels. In view of this finding and results from previous

(clinical) studies (3;7), the terminology "Intensive Insulin Therapy" introduced in 2001 (2) may be confusing, as it falsely suggests that insulin is the beneficial component of the intervention. However, the observation that the hyperglycemic-hyperinsulinemic group revealed most pronounced alterations regarding aortic NOS-activity and arginine levels, might indicate that a high insulin therapeutic regimen could be potentially deleterious if glucose levels are not controlled.

Some limitations of our study need to be highlighted. Firstly, as in critically ill humans (6), we did not succeed to reliably measure NOS and BH4 protein content by western blotting in our animal model, thus making it impossible to judge whether the intervention modified NOS or BH4 protein. Second, since critical illness reflects a complex entity, we cannot completely exclude other potential posttranslational modifiers of local NO bioavailability, such as oxidative stress. Third, as glucose intake inevitably differed among groups to independently achieve the different levels of the blood glucose and insulin, we cannot exclude an effect of the different caloric loads. However, caloric intake in all groups remained within the normal physiological range of rabbits. Fourth, measurement of NOS-activity and expression were performed on snap frozen biopsies, in which the heterogeneous cell population, with additional cell types apart from endothelial cells being present in these tissues, may have influenced the results. Fifth, we only performed our measurements in those animals that survived until day 7; hence we cannot exclude a selection bias. However, one would expect the most pronounced alterations in the animals dying before day 7, so that this selection bias is in favour rather against our conclusions. Sixth, since we had to limit the sample size due to ethical reasons the results might lack power to completely rule out minor effects of insulin levels. Finally, directly extrapolating from our animal model to human critical illness should be done with great caution.

As a conclusion, in our *in vivo* animal model of prolonged critical illness, prevention of excessive systemic NO release and preservation of physiological regional endothelial function was brought about by strict maintenance of normoglycemia. Factors contributing could be direct endothelial cell damage, direct effects on the enzyme activity, decreased substrate availability or decreased NO-induced inhibition. We could not detect any glycemia independent actions of insulin.

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