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 6

Glycemic control modulates arginine and asymmetrical dimethylarginine levels during critical illness by preserving dimethylarginine-dimethylaminohydrolase activity

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Abstract

Introduction
In the context of the hypercatabolic response to stress, critically ill patients reveal hyperglycemia and elevated levels of asymmetric-dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthases. Both hyperglycemia and elevated ADMA-levels predict increased morbidity and mortality. Tight glycemic control by intensive insulin therapy (IIT) lowers circulating ADMA-levels and improves morbidity and mortality. Methylarginines (MA) are released from proteins during catabolism. ADMA is predominantly cleared by the enzyme dimethylarginine-dimethylaminohydrolase (DDAH) in different tissues whereas its symmetric isoform (SDMA) is cleared via the kidneys. Hence glycemic control or glycemia-independent actions of insulin on protein breakdown and/or on DDAH-activity resulting in augmented ADMA-levels may explain part of the clinical benefit of IIT.

Methods
We investigated in our animal model of prolonged critical illness the relative impact of maintaining normoglycemia and of glycemia-independent action of insulin over 7 days in a 4-arm design on plasma and tissue levels of ADMA and SDMA, on proteolysis as revealed by surrogate parameters as changes of bodyweight, plasma urea/creatinine (U/C)-ratio and plasma levels of SDMA, and on tissue DDAH-activity.

Results
ADMA-levels remained normal in the 2 normoglycemic groups and increased in hyperglycemic groups. SDMA-levels in the investigated tissues remained largely unaffected. U/C-ratio indicated reduced proteolysis in all but normoglycemic/normal insulin animals. DDAH-activity deteriorated in hyperglycemic compared to normoglycemic groups. Insulin did not affect this finding independent of glycemic control action.

Conclusions
Maintenance of normoglycemia and not glycemia-independent actions of insulin maintained physiological ADMA plasma and tissue levels by preserving physiological DDAH-activity.

Introduction

As an integral part of the body‘s response to stress, critically ill patients uniformly reveal complex endocrine and metabolic changes (1). Hypercatabolism occurs, with protein breakdown from skeletal muscle and visceral structures. Concomitantly hyperglycemia is brought about by insulin resistance, increased gluconeogenesis and relative insulin deficiency, the so called "diabetes of injury" (2). The notion of hyperglycemia being a beneficial adaptation to stress, in order to provide substrates for vital organ function from endogenous stores, was recently challenged since it is associated with a number of life threatening complications and death (3). Moreover, strictly maintaining normoglycemia by intensive insulin therapy (IIT) improved morbidity and mortality of critically ill patients (4-6). Among other mechanisms (7), the modulation of nitric oxide (NO) metabolism emerges as a potentially important mechanism contributing to the clinical benefits (8-10).

NO is released by NO synthases (NOS), a family of enzymes converting the amino acid arginine as the sole substrate into NO and citrulline (11). NO modulates inflammation, coagulation, and vasomotor tone and thus microcirculatory blood-flow and trafficking of nutrients (12). During critical illness, it appears crucial to preserve physiological NO metabolism since both excessive and impaired NO-release are associated with impaired organ function and increased mortality. Indeed, NOS activity can be up-regulated in critical illness by a number of factors such as endotoxin or cytokines (13;14) with ensuing cardiovascular collapse (14;15). Of special interest, also hyperglycemia can cause this overwhelming NO production (16;17). However, reducing NO production via pharmacologically inhibiting NOS was shown to be deleterious since it impaired microcirculation, aggravated organ dysfunction and led to increased mortality (18). Also elevated levels of endogenous NOS-inhibitors, in particular asymmetric-dimethylarginine (ADMA), are found in critically ill patients and emerge as risk factors for organ failure and death (19;20). ADMA impairs endothelial function and NO-dependent vasorelaxation by both diminishing NOS-activity (21;22) and by competing with arginine for cellular transport leading to substrate depletion (23). It is synthesized when arginine residues in proteins are methylated by the action of protein arginine methyltransferases (PRMT). During proteolysis, ADMA and the symmetric isoform (SDMA) are released from the protein pool. Hence, since methylarginines (MA) are virtually not incorporated into de-novo synthesized proteins, the levels in tissue might reflect the overall metabolic activity and the protein turnover rate of cells (24;25). ADMA is eliminated in the cytosol via degradation by the enzyme dimethylarginine-dimethylaminohydrolase (DDAH). When intracellular ADMA-levels surmount the degradation capacity it is externalized via bidirectional working cationic amino acid transporters (CAT) (26). Circulating ADMA is predominantly cleared by DDAH in liver and kidney

(26) or to a minor extent excreted in the urine (27), whereas SDMA is predominantly cleared by renal excretion (28). Apart from impact on hemodilution or –concentration, elevated MA levels in plasma and tissues may thus result from both excessive protein breakdown and/or reduced clearance by diminished tissue DDAH-activity (ADMA) (29;30) and impaired renal function (SDMA) (28). However, impaired DDAH-activity might occur in context of kidney or liver failure (20).

Preventing hyperglycemia with IIT concomitantly lowers the plasma concentrations of ADMA in critically ill patients (9) and affects regional NO-metabolism (8;10;31). This effect on regional NO-metabolism emerges as a potential mechanism explaining part of the clinical benefits of IIT (8). Until now, it remains unclear whether an effect on regional NO-metabolism is brought about by modulating the substrate availability for NOS, hence plasma and tissue levels of arginine and MA, and whether this effect occurs as a result of modulating protein kinetics or MA-clearance. Moreover, since insulin has various actions besides its effect on blood glucose, it is of special interest whether the observed effects are directly brought about by insulin or result from the glycemic control that is obtained concomitantly.

In our animal model of prolonged critical illness, we therefore assessed the relative impact of glycemic control and glycemia-independent actions of insulin on levels of arginine and MA in plasma and myocardium, skeletal muscle, kidney, and liver biopsies. In addition, we evaluated surrogate markers of protein catabolism, measured DDAH-activity in the biopsies and assessed parameters of liver and kidney function.

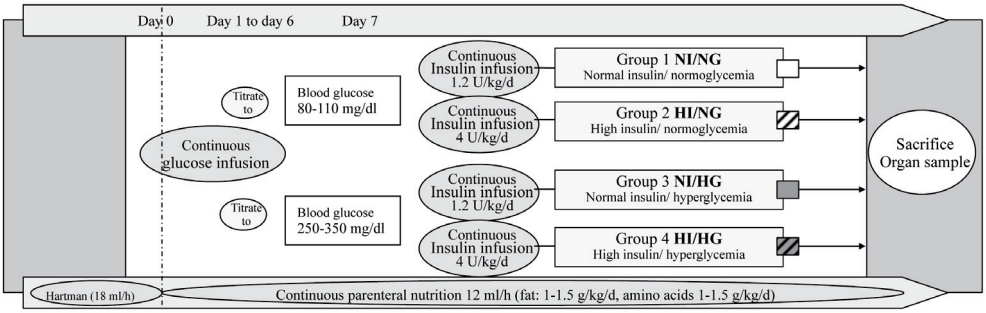
Materials and Methods

The protocol was approved by the University of Leuven ethical review board for animal research (protocol Nr. P 04058). 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.

In our animal model (32), metabolic and endocrine changes during a 7 days study-period reveal the typical biphasic course of acute and prolonged critical illness of the human counterpart (33). Moreover, the model provides the opportunity to manipulate plasma insulin levels and blood glucose independently as described in detail previously (10). Briefly (see Figure 1), in male, adult New-Zealand white rabbits endogenous insulin deficiency was induced by alloxan-monohydrate (150 mg/kg, Alloxan, Sigma-Aldrich, Bornem, Belgium), and critical illness was brought about by a third degree burn-injury of 20% body-surface-area. Via an indwelling central venous line continuous parenteral nutrition (Clinomel N7 containing 3.22 g/l arginine, Baxter, Clinitec, Maurepas Cedex, France) was administered to assure basal glucose, amino acid and fat

intake. Caloric intake as well as amino acid and fat intake (each 1-1.5 g/kg/d, arginine intake 0.1 g/kg/d) meet the physiologic requirements of adult rabbits (34). Additionally, we administered insulin (Actrapid, Novo Nordisk, Begsvaerd, Denmark) by means of a continuous fixed dose intravenous infusion to receive low and high physiologic plasma insulin levels, respectively. On each insulin level, we manipulated blood glucose by adjusting the speed of a supplementary intravenous glucose infusion (Glucose 50%, Baxter, Lessiness, Belgium) to receive either normoglycemia or hyperglycemia, respectively. Thus, four study groups resulted (figure 1): group 1: normal insulin levels and normoglycemia (NI/NG, n=8)), group 2: high insulin levels and normoglycemia (HI/NG, n=8), group 3: normal insulin levels and hyperglycemia (NI/HG, n=9), and group 4: high insulin levels and hyperglycemia (HI/HG, n=8). Samples from animals without alloxan injection and burn-injury served as "healthy controls" (n=8).

Figure 1. Flow chart of the experiment.



Insulin was continuously administered at a fixed dose from day 0 to day 7. The infusion rate of glucose 50% (G 50%) was adjusted to regulate glycemia from day 0 to day 7.

Blood glucose (glucose-oxidase method), electrolytes and haemoglobin were quantified in arterial whole 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 glycemia. 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 further analysis.
On day 7, animals were sacrificed and tissue samples of myocardium, skeletal muscle (quadriceps femoris), kidney and liver were taken, immediately snapfrozen in liquid nitrogen and stored at -80°C until analysis.

Assays
All analyses were conducted blinded to randomisation within a single assay run. All chemicals were derived from Sigma-Aldrich (Bornem, Belgium) unless indicated otherwise. Plasma insulin was measured by RIA using a guinea pig derived antibody as described elsewhere, within assay variation <10% (35) (kindly provided by R. Bouillon, KU Leuven, Belgium).

Protein content in the four investigated tissues was measured using Coomassie Protein Assay Reagent (Pierce Biotechnology, Erembodegem, Belgium) according to the users manual and a standard curve of bovine serum albumin as described previously (36).

Plasma levels of arginine, ADMA and SDMA

The concentrations of arginine, ADMA and SDMA were determined simultaneously by high-performance liquid chromatography (HPLC) as described previously (37;38). In brief, solid-phase extraction on polymeric cation-exchange columns was performed after addition of monomethylarginine as the internal standard. After derivatisation with ortho-phthaldialdehyde reagent containing 3-mercaptopropionic acid, analytes were separated by isocratic reversed-phase HPLC with fluorescence detection. Intra- and inter-assay coefficients of variation were 1.2 and 3.0%, respectively. The arginine over ADMA ratio was calculated.

Tissue levels of arginine, ADMA and SDMA

For the determination of arginine, ADMA and SDMA in myocardium, skeletal muscle, kidney and liver, tissue was homogenized with an OMNI-2000 Homogeniser (OMNI International, Waterbury, Conn., USA) in four volumes of sodium phosphate buffer (100 mM; pH 6.5). 250 µl of the tissue homogenate were mixed with 250 µl 1.2 M perchloric acid. After centrifugation (10 minutes at 2,000 g at 4ºC), 200 µl of the supernatant was mixed with 400µl of 0.5 M di-sodium hydrogen phosphate, 100 µl of the internal standard solution, and 400 µl water. This mixture was subjected to solid-phase extraction, derivatisation and chromatography as described above (37;38).

Tissue DDAH-activity

Tissue DDAH-activity was determined by measurement of citrulline formation during incubation of tissue homogenates with excess of ADMA. Tissue samples were homogenized with four volumes of sodium phosphate buffer (100 mM; pH 6.5) at 4°C with an OMNI-2000 Homogeniser and subjected to a dual centrifugation procedure (10 minutes at 2,000 g, followed by 30 minutes at 10,000 g, both at 4°C). 160 µl of the supernatant were mixed with 240 µl of a 4 mM solution of ADMA in sodium phosphate buffer (100 M; pH 6.5) containing protease inhibitors (Complete protease inhibitor cocktail from Roche Applied Science, Mannheim, Germany). Before and after incubation for 2 h at 37 °C, the reaction was stopped and proteins were precipitated by transferring 200 µl of the incubation mixture to vials containing 8 mg sulfosalicylic acid. After vortex-mixing, the vials were centrifuged (10 min at 3,000 g at 4ºC) and citrulline in the clear supernatant was measured by high-performance liquid chromatography as described previously (39). The increase of citrulline concentration during incubation was used to calculate DDAH-activity (expressed

as nmol/min/g wet weight of tissue). Control incubations were performed in the absence of either tissue homogenate or ADMA, and in the presence of an excess citrulline, as previously described (20).

Organ function parameters

By commercial kits, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in plasma according to the guidelines of the International Federation of Clinical Chemistry. Creatinine in plasma was measured by the Jaffé method, and urea in plasma was measured by means of a kinetic UV assay with equipment specific reagents. Modular Roche and specific reagents by Roche (Roche/Hitachi, Bern, Swizerland; A.M.L bvba, Antwerp, Belgium) were used according to the users manual. As proteine intake was equal in all groups, the ratio of plasma urea over plasma creatinine (U/C-ratio) was calculated and the changes from baseline (delta U/C-ratio) determined as a surrogate marker of catabolism (40). An increase of this ratio is suggestive for increased and a decline for reduced protein breakdown.

Statistical analysis

Results are expressed as mean ± SEM. Data are presented as box plots; the central line indicates the median, the box the interquartile range, and the whiskers the 10th and 90th percentiles. Data were analysed by multifactorial ANOVA, and Fisher`s Protected Least Significant Difference post hoc testing (FPLSD). For non-normally distributed data Kruskal-Wallis and Mann-Whitney-U tests were used. Correlation analysis was performed by linear or logarithmic regression (Pearson Coefficient). A two tailed p-value <0.05 was considered significant. The statistical analyses were done with StatView 5.0.1.

Results

We described previously that in our model blood glucose and insulin levels were manipulated independently from each other (10). Glucose intake inevitably differed among groups to achieve this (table 1); protein and lipid intake did not differ significantly. Protein content per gram of tissue did not differ among groups and from healthy controls in any of the investigated tissues. Plasma levels of electrolytes, haemoglobin and surrogate parameters of cardiac preload as given in (10) did not differ significantly among groups largely excluding intravascular fluid volume contraction or expansion.

In plasma, the arginine levels were lower in the hyperglycemic groups compared to normoglycemic groups and healthy controls. Grouping both hyperglycemic and normoglycemic groups, ADMA plasma levels were elevated in hyperglycemic animals compared to normoglycemic animals (Figure 2). Consequently, the arginine over ADMA ratio in plasma was significantly lower in hyperglycemic groups compared to normoglycemic animals. The pattern was largely

comparable on day 3 and day 7. Insulin evoked no glycemia-independent actions.

Table 1. Glucose intake, plasma insulin levels, blood glucose, changes in body-weight and protein-content of the investigated tissues.

	NI/NG	*HI/NG*	*NI/HG*	***HI/HG***
Glucose intake (g/d) [a]	27.4 (±1.9)	47.2 (±2.4)	64.3 (±2.8)	75.9 (±3.1)
Plasma insulin (mU/l) [b]	43.3 (±3.4)	188 (±16.7)	47.3 (±3.5)	199.8 (±15.9)
Blood glucose (mg/dl) [c]	93.0 (±3.4)	85.2 (±3.5)	328 (±11.3)	314 (±10.6)
BW-changes (g/7days) [d]	-21.8 (±146.4)	-87.5 (±32.4)	-36.1 (±75.7)	140.6 (±34.3)
Tissue protein-content (mg protein /g tissue)				
Myocardium [d]	184.08 (±3.0)	191.10 (±8.5)	182.73 (±4.2)	173.05 (±4.3)
Muscle [d]	141.95 (±4.9)	138.98 (±5.1)	140.27 (±6.7)	143.39 (±9.4)
Kidney [d]	88.6 (±5.6)	76.49 (±4.3)	83.56 (±6.5)	72.33 (±3.9)
Liver [d]	137.81 (±3.9)	120.43 (±8.1)	118.79 (±12.6)	112.50 (±8.7)

Values are mean (±SEM) over 7 days, respectively, and mean (±SEM) in tissue samples on day 7. [a]Glucose intake differed significantly among groups ($P < 0.03$).
[b]Plasma insulin levels in both normal insulin groups were equal ($P = 0.9$) but different ($P < 0.0001$) from both high insulin groups. Insulin levels in both high insulin groups did not differ ($P = 0.5$).
[c]Glycemia in both normoglycemic groups was equal ($P = 0.6$) but different ($P < 0.0001$) from both hyperglycemic groups. Blood glucose levels in both hyperglycemic groups did not differ ($P = 0.4$).
[d]No significant difference among groups.

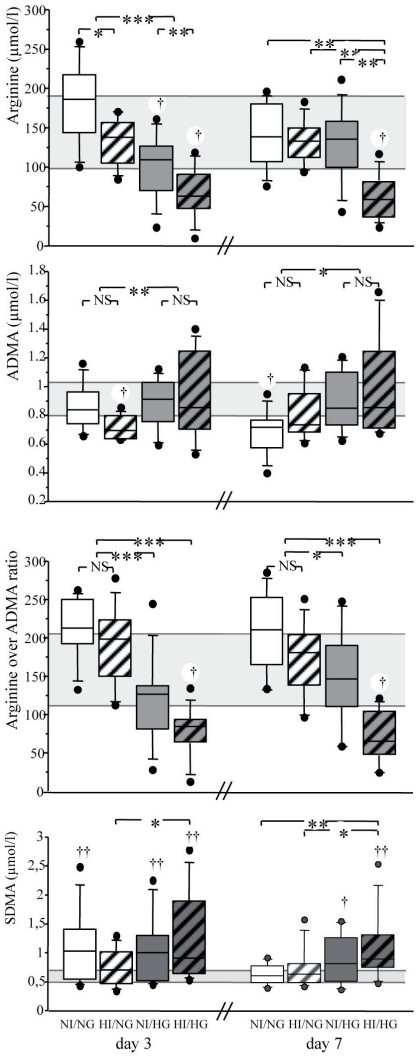
The pattern of the tissue levels of ADMA was similar in all investigated tissues, with higher ADMA levels in both hyperglycemic groups as compared with normoglycemic groups (Figure 3). ADMA in liver was elevated in sick animals compared to healthy controls (p=0.007), most pronounced in HI/HG animals. The arginine over ADMA ratio was reduced in both hyperglycemic groups as compared with normoglycemic groups and healthy controls in all investigated tissues. Insulin evoked no glycemia-independent actions. SDMA remained unaffected by the intervention in the investigated tissues.
The arginine over ADMA ratio in all tissues correlated with the ratio in plasma ($p<0.0003$, all $R^2 >0.34$). Plasma SDMA correlated with SDMA in myocardium, kidney and liver ($p<0.0001$, all $R^2 >0.4$), but not with SDMA in skeletal muscle ($p=0.228$, $R^2 =0.018$) (data not shown).
The U/C-ratio increased from baseline to day 7 (p=0.02) in NI/NG animals whereas in HI/NG, NI/HG and HI/HG animals, this ratio decreased ($p<0.001$) (Figure 4).

The DDAH-activity in myocardium and skeletal muscle did not differ among groups and was similar to healthy controls (Figure 5). In kidney and liver both hyperglycemic groups revealed lower DDAH-activity than both normoglycemic groups. In liver, both normoglycemic groups revealed higher DDAH-activity than healthy controls ($p<0.05$). Insulin evoked no glycemia-independent actions.

The plasma levels of ADMA negatively correlated with DDAH-activity in kidney and in liver (Figure 6). Likewise, the arginine over ADMA ratio in plasma correlated with the DDAH-activity in kidney and liver. In myocardium and muscle no correlation between DDAH-activity and plasma levels of arginine and ADMA was found (data not shown).

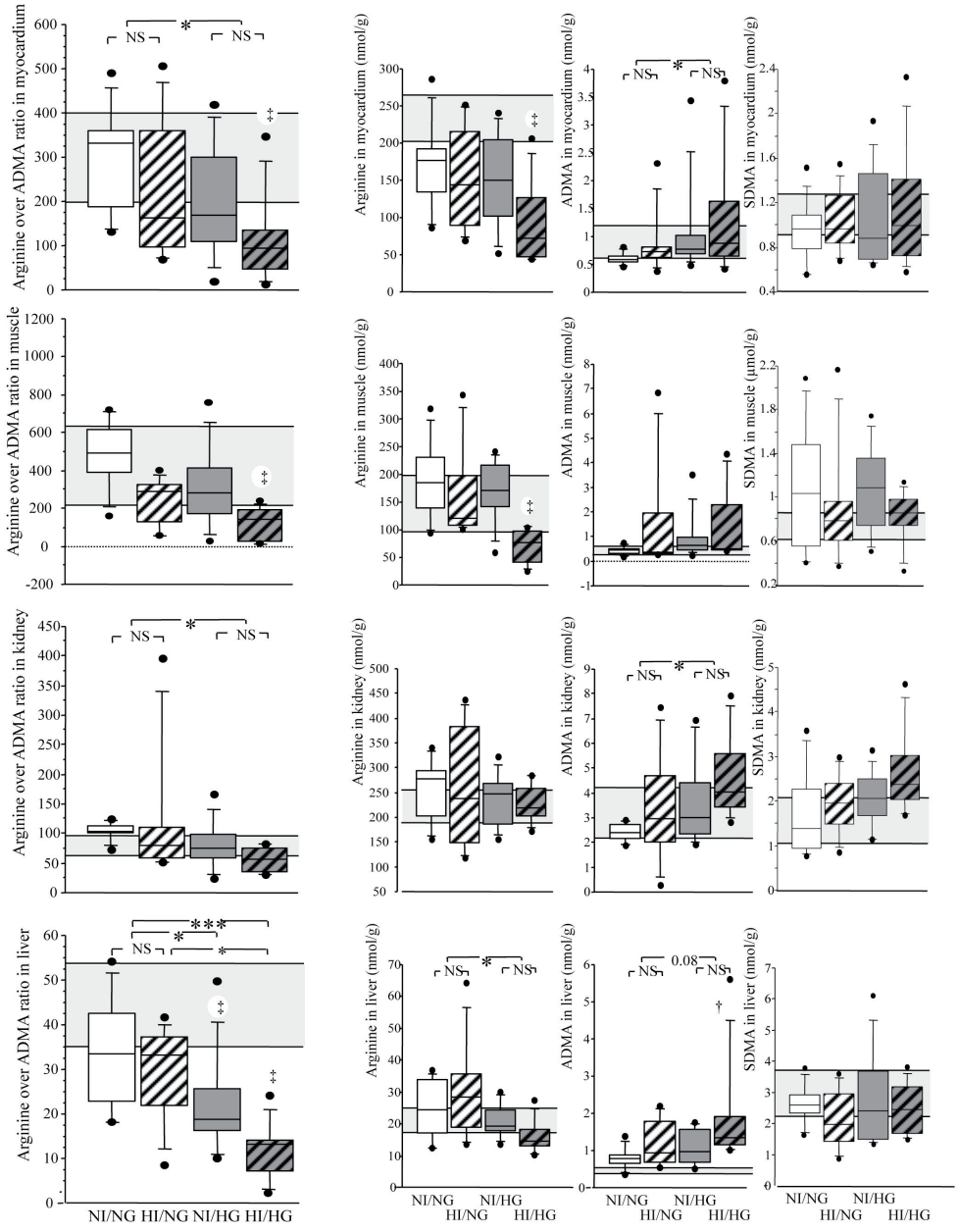
We previously reported altered liver and kidney function in the hyperglycemic groups in our model (10). DDAH-activity in liver correlated with the plasma markers of liver function, and DDAH-activity in kidney and SDMA in plasma correlated with plasma creatinine (Figure 7).

Figure 2. Plasma levels of arginine, ADMA, arginine over ADMA ratio and SDMA on day 3 and 7.



Levels of arginine (upper panel), ADMA (second panel), arginine over ADMA ratio (third panel), and SDMA (lower panel) in plasma on day 3 (left column) and day 7 (right column), grey area reflects range in healthy controls. *p<0.05, **p<0.01, ***p<0.001, †: p<0.05, ††: p<0.001 versus healthy controls, NS: not significant.

Figure 3. Tissue levels of the arginine over ADMA ratio, arginine, ADMA and SDMA.

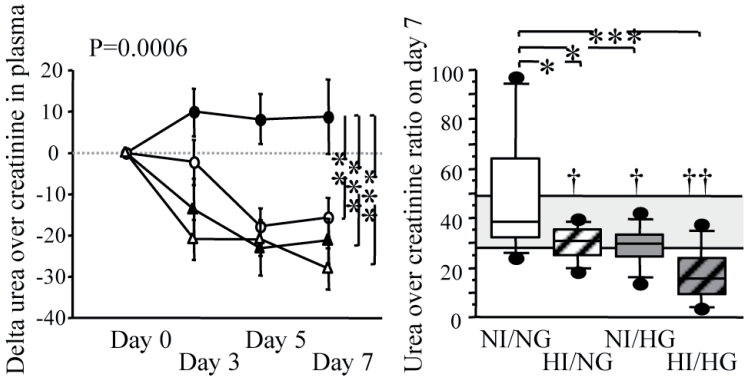


Tissue levels of the arginine over ADMA ratio (left column), arginine (left middle column), ADMA (right middle column) and SDMA (right column) in myocardium (upper row), skeletal muscle (second row), kidney (third row), and liver (lower row); grey area reflects range in healthy controls. *p<0.05, ***p<0.001, †p<0.01 vs. healthy controls, ‡p<0.05 vs. all other groups and healthy controls. NS: not significant.

Discussion

In this model of prolonged critical illness, preventing hyperglycemia and not glycemia-independent actions of insulin maintained physiological arginine and ADMA levels in plasma and tissue biopsies of myocardium, skeletal muscle, kidney and liver. This was most likely explained by preserved breakdown of ADMA via maintaining physiological DDAH-activity rather than by an effect on MA release via protein catabolism.

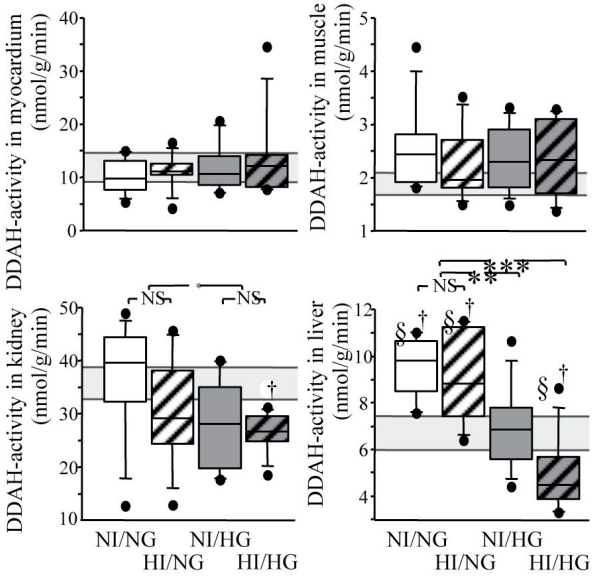
Figure 4: Changes of the urea over creatinine ratio over time (left panel) and urea over creatinine ratio among groups on day 7 (right panel). Grey area reflects range in healthy controls.



Filled circle: NI/NG, open circle: HI/NG, filled triangle: NI/HG, open triangle: HI/HG, *p<0.05, **p<0.01, ***p<0.001, †p<0.01 versus healthy controls, †† p<0.001 versus healthy controls.

In our experiment, plasma ADMA-levels in both hyperglycemic groups were elevated compared to both normoglycemic groups, irrespectively of the insulin levels. Concomitantly to increased ADMA, arginine levels in plasma were decreased in both hyperglycemic groups compared to both normoglycemic groups. This resulted in a pronounced decrease in the arginine over ADMA ratio in hyperglycemic groups. The pattern of the arginine and ADMA-levels in plasma largely reflected the pattern in the investigated tissue biopsies. Also in the tissues, consistently, although not always reaching statistical significance, ADMA-levels were elevated in hyperglycemic compared to normoglycemic groups and the arginine over ADMA ratio was diminished in both hyperglycemic groups. Since ADMA is not reincorporated into proteins (24;25) our findings are compatible with the notion that plasma levels of ADMA mainly reflect cellular spill-over of the amount of ADMA that surmounts intra-cellular clearance capacity (25;41).

Figure 5: DDAH-activity in myocardium (left upper panel), skeletal muscle (right upper panel), kidney (left lower panel) and liver (right lower panel). Grey area reflects range in healthy controls.



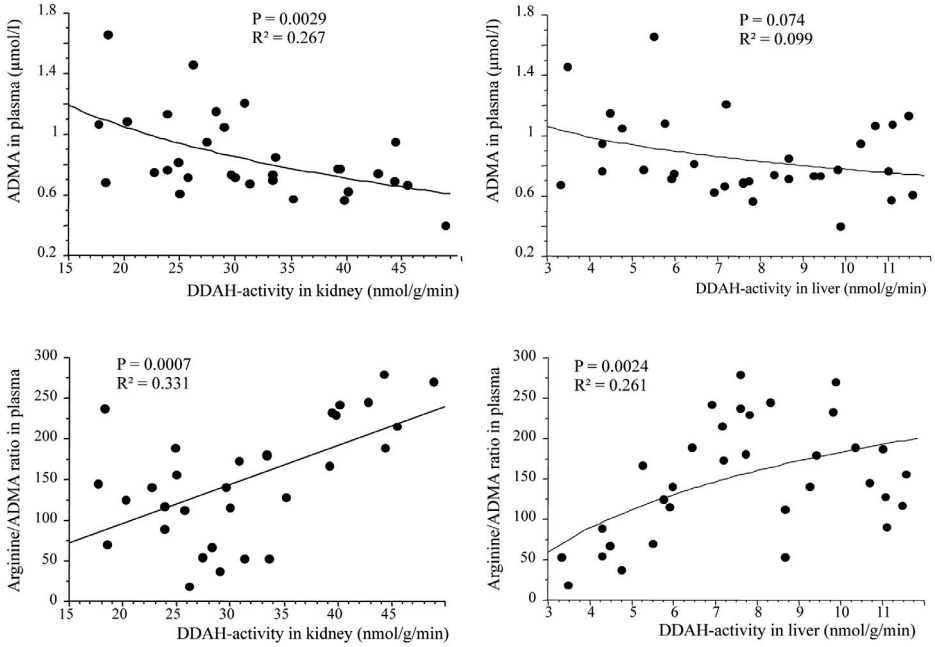
*p<0.05, **p<0.01, ***p<0.001, NS: not significant, † p<0.05 versus healthy controls.

In human diabetes mellitus elevated ADMA-levels occur in the context of insulin resistance (42) and hyperglycemia (43) rendering patients at risk for cardiovascular complications (44). Equivalently, in human critical illness increased ADMA-levels occurred in plasma already on day 2 after the onset of the disease (9), and emerged as independent predictors of organ dysfunction and mortality (19). Strictly maintaining normoglycemia by IIT was shown to prevent elevated ADMA-levels. In view of our current results and the findings in a rat model of type II diabetes mellitus (13) this is most likely brought about by strict glycemic control and not by glycemia-independent actions of insulin. However, not only preventing increased ADMA-levels but the preservation of a physiological balance between arginine and ADMA by maintaining normoglycemia appears important, since the arginine over ADMA ratio is the major determinant of NOS-activity in tissues (10;45). Notably, neither maintaining normoglycemia nor insulin increased this ratio above normal values. Elevated arginine over ADMA ratio could be considered potentially deleterious, as it might lead to overwhelming NO production. Hence, maintaining normoglycemia during critical illness not only reduces ADMA-levels but largely preserves the physiological regulation of substrate availability for NOS (10). The latter property appears crucial for survival of critically ill patients since both

overwhelming NO production with ensuing cardiovascular collapse, and local NO deficiency with altered endothelial function and disturbed microcirculation, are detrimental (18).

Elevated ADMA-levels during critical illness may either result from increased generation or from impaired breakdown. In fact, MA like ADMA and SDMA are extensively released from the intra-cellular protein pool during high protein turn-over, so that high levels of ADMA (25) and SDMA (24) in plasma and tissues might be expected during hypercatabolism. Assuming that critical illness evokes hypercatabolism, protein breakdown from organs and tissues with a known high protein turn-over rate, such as the liver and muscle, is expected (46). In liver, but not in the other tissues, we found that the ADMA-levels in all sick groups were moderately increased as compared with healthy controls, with hyperglycemic animals consistently revealing the highest ADMA-levels. Meanwhile SDMA levels did not increase in any group in any of the tissues, making it unlikely that elevated ADMA and differences among groups result from an effect on ADMA release from protein breakdown (24). Moreover, the U/C-ratio, roughly reflecting protein breakdown from muscle (40), was reduced with the interventions in all but the NI/NG group, indicating reduced catabolism. In the NI/NG-animals, the U/C-ratio increased with time, suggesting increased catabolism. These animals, however, revealed the lowest circulating and muscle ADMA-levels. Although insulin is considered an anabolic hormone reducing protein breakdown and thereby, hypothetically, causing less ADMA release (20), we could not detect any glycemia-independent actions of insulin on protein breakdown in muscle and on ADMA-levels. Also, protein content in tissues and body-weight did not change in any of the groups during the experiment, largely excluding profound protein depletion and starvation (34). The remarkable variance in body-weight changes can be interpreted as a result from variable bladder and gastric distension, an inconsistent amount of oedema in the burn wound and a variable degree of ascites. However, it remains unclear why the U/C-ratio indicates catabolism in HI/HG animals only although the other parameters did not reveal any evidence for increased catabolism in this group. Furthermore, in the other groups U/C-ratio decreased indicating anabolism although the other surrogate parameters did not reveal any evidence for this notion. A possible explanation might be seen in the bias of the measures by fluid load or an impact of organ dysfunction. However, also in the clinical study of IIT, there was no clear evidence that glycemic control and/or insulin, in the doses that were used to maintain normoglycemia, affected catabolism (33). Conclusively, since our experimental setting was not designed accordingly it does not allow to circumstantially and precisely define minor effects of the interventions on catabolism or anabolism. Though, our methodology allows the statement that the impact of glycemic control on arginine and ADMA-levels is not likely explained by effects on MA generation via protein breakdown.

Figure 6: Correlation of the DDAH-activity in kidney (left column) and liver (right column) with plasma levels of ADMA (upper row) and arginine over ADMA ratio (lower row).



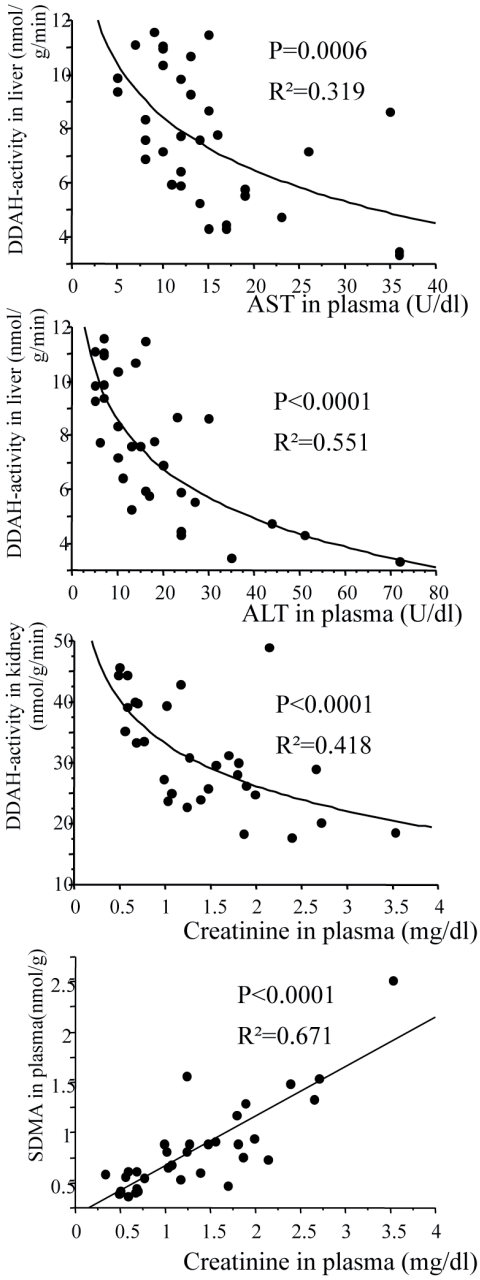
In contrast, it appears that blood glucose control affected ADMA-levels via reduced ADMA-clearance. ADMA is directly cleared by the enzyme DDAH in those tissues where it is generated. The ADMA quantity which surmounts tissue clearance capacity is externalised from the cells and appears in the circulation. From the circulation, ADMA is cleared by DDAH in kidney and liver, and to a minor extent by direct renal excretion (25). In a physiological control loop, DDAH-activity is up-regulated when ADMA-levels increase (26). We found in our experiment that in myocardium and muscle tissue of critically ill animals, DDAH-activity was largely preserved at the level of healthy controls. As in the presence of elevated ADMA levels, elevated DDAH activity would be required in the hyperglycemic groups, the physiologic control loop may be impaired by hyperglycemia in these tissues. More clearly this is the case in kidney and liver, as DDAH-activity in these tissues was frankly low in both hyperglycemic groups as compared with healthy controls and normoglycemic groups, in the presence of elevated ADMA. In both normoglycemic groups, however, the control loop of ADMA and DDAH-activity appeared intact since in the liver biopsies of both normoglycemic groups moderately elevated ADMA-levels and a concomitantly increased DDAH-activity was present. DDAH-activity in kidney and liver appears to be the major determinant of ADMA clearance from the plasma and for the preservation of a physiologic arginine over ADMA balance (25;26;29), as

indicated by the correlation of DDAH-activity in these tissues with plasma arginine and ADMA.

Hyperglycemia appeared to downregulate DDAH-activity in our model of critical illness. This is in line with what has been observed in cultured endothelial cells (47) and models of type II diabetes mellitus (48). Also in these studies, impaired DDAH-activity and not protein turn-over appeared to increase ADMA-levels. A potential reason for impaired DDAH-activity with hyperglycemia could be glycation of the DDAH-protein (49) or increased oxidative stress (7;48) as DDAH is an oxidant-sensitive enzyme (48). Insulin might theoretically ameliorate ADMA-clearance by affecting amino acid transporter-systems whereby increasing ADMA uptake into organs that eliminate ADMA (50). However, in our model we could not detect any glycemia-independent effect of insulin on DDAH-activity or ADMA levels in tissue or plasma.

Reduced DDAH-activity has been reported in a number of medical conditions, especially when kidney and liver function are impaired (20). Also in our experiment, DDAH-activity in kidney and liver correlated negatively with the organ function parameters. Hence, reduced DDAH-activity and hereby increased ADMA levels may result form organ failure. Unfortunately, we could not distinguish whether impaired DDAH-activity and increased ADMA levels were cause or consequence of organ dysfunction. However, SDMA is mainly cleared via renal excretion and may serve as a marker for renal dysfunction in humans (28). Also in our model, plasma-SDMA correlated well with plasma creatinine. Since SDMA levels in the tissues are not elevated largely excluding an effect of our interventions on SDMA-release, slightly elevated SDMA in plasma of HI/HG animals on day 7 should most probably be seen in the context of kidney dysfunction.

Figure 7: Correlation of the DDAH-activity with parameters of organ function. DDAH-activity in liver versus plasma AST (upper panel) and ALT (upper median panel), and DDAH-activity in kidney versus plasma creatinine (lower median panel). Correlation of plasma SDMA versus plasma creatinine (lower panel).



The limitations of our study need to be addressed. Firstly, extrapolating from our animal model to the complex entity of human critical illness should be done with great caution. Secondly, protein content and U/C-ratio can be considered as valid global markers of catabolism since protein intake did not differ among groups and kidney function remained stable the last days of the experiment (40). However, they are rough, not very specific and accurate markers of protein catabolism that are prone to several confounders. Most important, they can be influenced by organ dysfunction like e.g. renal failure. Hence, a subtle effect of maintaining normoglycemia or glycemia-independent actions of insulin on ADMA release from protein breakdown cannot completely be excluded. The accurate assessment of protein turnover would require the use of stable isotopes. Thirdly, since glucose intake inevitably differed among groups to obtain the independent manipulation of the blood glucose and insulin levels, we cannot exclude a contribution to our findings from the different caloric loads. However, since caloric intake in all groups remained in the range known as "normal" and physiologic for rabbits (34), we can at least exclude effects of starvation or overfeeding. Finally, although not reaching statistical significance, animals of the HI/HG group seem to reveal worse results in almost all investigated parameters as compared to the other groups. However, due to ethical reasons the numbers of animals per group had to be limited and might not suffice to completely rule out a minor glycemia independent effect of insulin during hyperglycemia.

In conclusion, in our in-vivo model of prolonged critical illness, maintenance of normoglycemia and not glycemia-independent actions of insulin maintained physiological ADMA levels and arginine over ADMA ratios in plasma and tissues by preserving physiological DDAH-activity.

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