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Hyperglycemic kidney damage in an animal model of prolonged critical illness

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Acute kidney injury frequently complicates critical illness and increases mortality; maintaining normoglycemia with insulin has been shown to reduce the incidence of intensive care unit (ICU)–acquired kidney injury. Here we tested the mechanisms by which this intervention might achieve its goal, using a rabbit model of burn-induced prolonged critical illness in which blood glucose and insulin were independently regulated at normal or elevated levels. Hyperglycemia caused elevated plasma creatinine and severe morphological kidney damage that correlated with elevated cortical glucose levels. Renal cortical perfusion and oxygen delivery were lower in hyperglycemic/hyperinsulinemic rabbits, compared to other groups, but this did not explain the elevated creatinine. Mitochondrial respiratory chain activities were severely reduced in the hyperglycemic groups (30–40% residual activity), and were inversely correlated with plasma creatinine and cortical glucose. These activities were much less affected by normoglycemia, and hyperinsulinemia was not directly protective. Mitochondrial damage, evident at day 3, preceded the structural injury evident at 7 days. Our study found that hyperglycemia evoked cellular glucose overload in the kidneys of critically ill rabbits, and this was associated with mitochondrial dysfunction and renal injury. Normoglycemia, independent of insulinemia, protected against this damage.

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The development of acute kidney injury (AKI) is a frequent complication of critical illness. Depending on the patient population and diagnostic criteria used, its prevalence ranges from 1 to 25%.[1,2] In a large, observational, multinational study, 5.7% of the patients had AKI at some time during their stay in the intensive care unit (ICU), ranging from 1.4 to 25.9% across centers.[3] The development of AKI requiring renal replacement therapy is associated with a high risk of death, generally above 50%.[1,3,4] Consensus has recently been achieved on classifying AKI based on the RIFLE (Risk of renal dysfunction; Injury to the kidney; Failure of kidney function; Loss of kidney function; and End-stage kidney disease) criteria,[1] shown to correlate with hospital mortality and length of ICU and hospital stay.[5]

Once severe AKI has developed, supporting the patient with renal replacement therapy to bridge time to spontaneous recovery is the only available option to prevent death.[4] Hence, prevention of AKI is crucial, but until recently, only hemodynamic support and avoidance of nephrotoxic substances had shown to be effective.[4,6] In two large prospective randomized clinical studies, we showed that strict control of blood glucose to normal levels with intensive insulin therapy reduced the incidence of ICU-acquired kidney injury compared with only treating excessive hyperglycemia above 215 mg per dl, besides other clinical benefits culminating in reduced risk of death.[7–9] Renal protection appeared more pronounced in surgical than in medical critically ill patients.[10] Other (randomized) studies and meta-analyses on the effects of insulin therapy yielded controversial results.[11–21] Generally, however, studies that were unable to detect clinical benefit already started with lower glucose levels in the conventional control group than did the Leuven studies. This may suggest that prevention of excessive hyperglycemia is what evokes the benefit, although the combination with inadequate glucose monitoring and poor achievement of the glycemic targets may have a role.

Two components have been implicated in the acutely decreased glomerular filtration rate in AKI.[2] These are a vascular component, comprising intrarenal vasoconstriction

and endothelial damage, and a tubular component, comprising tubular cellular injury and obstruction. The proximal tubule seems particularly sensitive to mitochondrial injury, which may have an important role in AKI. Indeed, the solute transport activities of the kidney require substantial energy production, and thus mitochondrial activity.[22] We previously showed a protective effect of intensive insulin therapy on the vascular endothelium and (hepatocytic) mitochondria of critically ill patients.[23,24] This study was designed to gain insight into the mechanisms of renal damage and protection with intensive insulin therapy during critical illness. In prolonged (7 days) critically ill rabbits from a previously reported experimental study,[25] we therefore investigated the separate effect of preventing excessive hyperglycemia and associated toxicity versus increasing insulin levels on renal perfusion, oxygen delivery, and mitochondrial function in relation to functional and structural kidney damage. These analyses were complemented with a new experimental study where we analyzed mitochondrial function and renal structural integrity earlier in the disease course (after 3 days of critical illness).

RESULTS

Blood glucose and insulin

According to the protocol, blood glucose and insulin levels were independently manipulated to normal or high levels for 7 days, resulting in four experimental groups (Table 1): normoinsulinemia/normoglycemia (NI/NG), hyperinsulinemia/normoglycemia (HI/NG), normoinsulinemia/hyperglycemia (NI/HG), and hyperinsulinemia/hyperglycemia (HI/HG).[25] To reach these targets, it was inevitable that glucose intake differed among the groups ($P<0.05$), however, always within the physiological range.[25,26] Amounts of infused lipids and amino acids were physiological and comparable in all groups.[25]

Survival and kidney injury

We previously showed that hyperglycemic animals had a higher risk of dying before day 7 (NI/HG, 5/14 = 35.7%; HI/HG, 7/15 = 46.7%) than normoglycemic rabbits (both NI/NG and HI/NG, 1/9 = 11.1%, $P=0.03$).[25]

On day 7 of critical illness, plasma creatinine levels were within the healthy reference range in the two normoglycemic groups, but significantly elevated in the two hyperglycemic groups (Figure 1a).[25] This reflected a median (interquartile range, IQR) fold change from baseline of 0.95 (0.66–1.72) for NI/NG, 1.14 (0.72–1.81) for HI/NG, 2.08 (1.19–2.37) for NI/HG, and 2.51 (1.51–3.88) for HI/HG. Hyperinsulinemia was not renoprotective.

Histological examination at this time point indicated clear morphological differences among the groups ($P=0.0006$) (Figure 1b). Observed abnormalities included dilatation of the tubules, flattening or loss of tubular epithelium, and the presence of intraluminal debris and/or calcifications

Table 1 | Blood glucose control and insulin levels

	NI/NG	HI/NG	NI/HG	HI/HG
Blood glucose (mg per dl)				
Baseline	151.0 ± 6.2	143.4 ± 12.2	149.5 ± 12.6	165.3 ± 9.4
Days 1–7	92.7 ± 1.9	84.5 ± 5.2	328.2 ± 16.8	314.3 ± 11.6
Plasma insulin (mU/l)				
Baseline	22.6 ± 5.2	40.8 ± 17.7	17.3 ± 3.0	14.8 ± 2.7
Days 3–7	43.3 ± 4.1	175.8 ± 13.1	47.3 ± 5.2	199.8 ± 25.9

HI/HG, hyperinsulinemia/hyperglycemia; HI/NG, hyperinsulinemia/normoglycemia; NI/HG, normoinsulinemia/hyperglycemia; NI/NG, normoinsulinemia/normoglycemia. Blood glucose and insulin levels were independently manipulated according to the study design.[25] Blood glucose and plasma insulin levels are presented as mean ± s.e.m. To convert glucose to mmol/l, multiply by 0.0551.

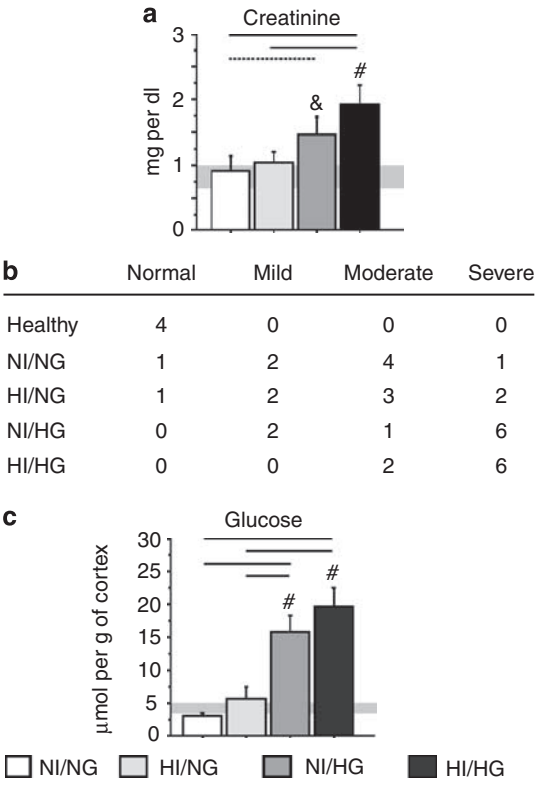

Figure 1 | Renal damage and glucose levels. (**a**) Day 7 creatinine levels. To convert values for creatinine to μmol/l, multiply by 88.4. NI/NG ($n=8$), HI/NG ($n=8$), NI/HG ($n=9$), and HI/HG ($n=8$). Data are presented as mean and s.e.m. The gray box indicates the mean ± s.e.m. of healthy reference ranges ($n=4$). (**b**) Scoring of morphological lesions on periodic acid-Schiff staining. Lesions were scored 'mild' when a minority of tubules with flattening and/or loss of the tubular epithelium and with intraluminal debris were present only or mainly in the medullary rays; 'moderate' when a minority of tubules with flattening and/or loss of the tubular epithelium and with intraluminal debris, whether or not calcified, were present in the medullary rays and convoluted part of the cortex; 'severe' when a conspicuous number of tubules with flattening and/or loss of the tubular epithelium and with intraluminal debris, whether or not calcified, were present in the medullary rays and convoluted part of the cortex and/or cortical necrosis was present. Numbers indicate the number of animals in each class. (**c**) Glucose levels in the renal cortex are expressed per g wet weight. NI/NG ($n=8$), HI/NG ($n=8$), NI/HG ($n=9$), and HI/HG ($n=8$). Data are presented as mean and s.e.m. The gray box indicates the mean ± s.e.m of healthy reference ranges ($n=8$). $^{\#}P\leqslant0.05$; &: $0.05<P\leqslant0.1$ compared with healthy reference ranges. Solid lines: $P\leqslant0.05$; dashed lines: $0.05<P\leqslant0.1$ between critically ill groups.

(Figure 2). With mild injury (observed in all sick groups), these abnormalities were mainly present in the medullary rays, whereas an increasing number of tubules in an extended area (the medullary rays and convoluted part of the cortex) were involved when damage was more severe (Figure 1b). Necrotic areas were rare ($n=2$). These microscopic lesions were more severe in the two hyperglycemic than in the two normoglycemic rabbit groups ($P=0.02$) (Figure 1b). Glomeruli were not affected.

Kidney glucose content

Critically ill rabbits subjected to elevated circulating glucose levels showed substantial cellular glucose overload in the renal cortex on day 7 (four-fold higher than the healthy reference range, Figure 1c). When circulating levels of glucose were maintained normal, cortical glucose levels were also normal. Cortical glucose levels correlated positively with plasma creatinine ($R=0.546$, $P=0.0005$).

Oxygen supply

We previously showed that cardiac output at the end of the 7-day experimental period was comparable for all groups.[25] At that time, whole-body arterial oxygen content (CaO_2) and oxygen delivery (DaO_2) were comparably low in the four sick groups (data not shown). Perfusion, estimated by the accumulation of microspheres, and oxygen delivery to the cortex, was lower than the healthy reference range only in HI/HG rabbits (Figure 3). Plasma creatinine did not correlate with oxygen delivery to the cortex ($R=-0.206$; $P=0.3$). Rather than oxygen supply, the tissue level of glucose in the kidney correlated with injury, as indicated by plasma creatinine. Indeed, when both variables were entered in a stepwise regression analysis, only glucose was maintained in the statistical model ($R=0.592$; $P=0.0004$).

Mitochondrial function

At day 7, the activity of all renal cortex mitochondrial respiratory chain enzyme complexes, except complex IV, was low in the two groups of hyperglycemic rabbits, with residual activities of 30–40% of healthy reference values (Figure 4). Some enzymes appeared compromised also in normoglycemic sick rabbits, but to a much lesser degree than in hyperglycemic rabbits. Activity of complexes I, II, III, and V was ~2-fold higher in normoglycemic than in hyperglycemic sick rabbits. The activity of complex IV was upregulated by illness, as seen in normoglycemic animals, but this effect was counter-acted by hyperglycemia. Hyperinsulinemia did not protect the mitochondria.

The level of activity of the affected mitochondrial enzymes showed a strong inverse correlation with degree of kidney injury (plasma creatinine), cortical glucose content, and to a lesser degree renal cortex oxygen supply (Table 2). Importantly, however, cortical glucose content appeared the key determinant for enzyme activity when both glucose content and oxygen supply were entered in a stepwise regression analysis (Table 3a). Similarly, mitochondrial damage, and not

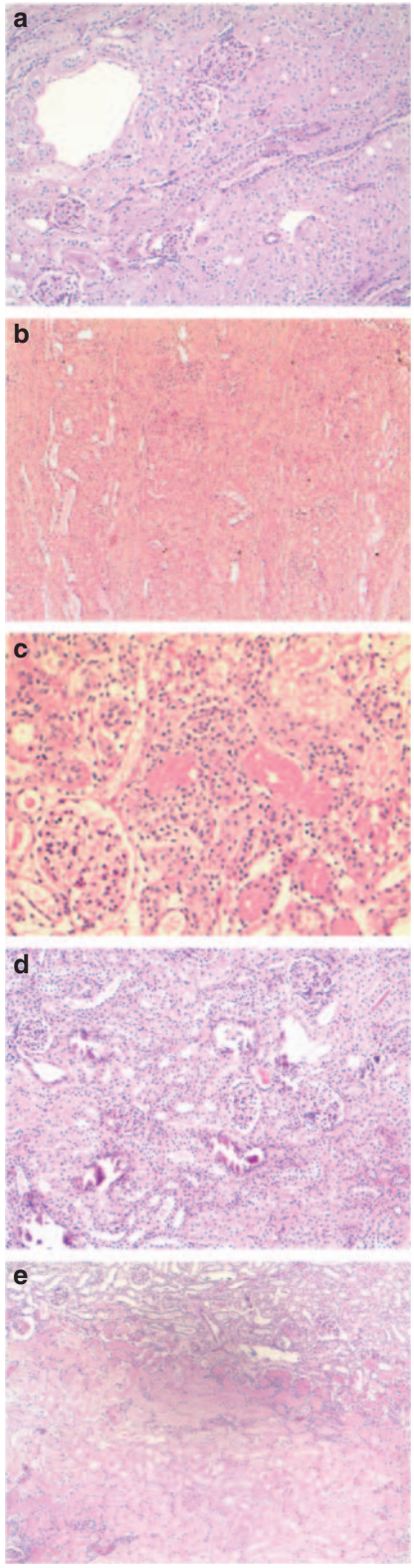

Figure 2 | PAS staining of paraffin sections of kidney. (**a**) Normal morphology, (**b**) tubules with flattening and/or loss of the tubular epithelium and filled with debris in the medullary rays, (**c**) tubules in convoluted part of the cortex with flattening and/or loss of the tubular epithelium and filled with debris, (**d**) tubules in the convoluted part of the cortex with flattening and/or loss of the tubular epithelium and with calcifications in the lumen, and (**e**) cortex necrosis. Original magnification ×50 (**b**, **e**), ×100 (**a**, **d**), and ×200 (**c**).

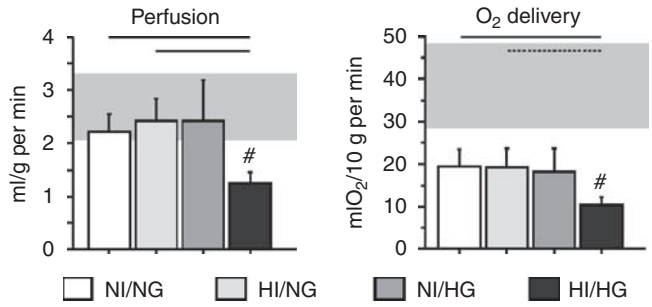

Figure 3 | Renal perfusion and oxygen supply. Oxygen supply to the renal cortex was calculated as the product of perfusion estimated by the microspheres and arterial oxygen content CaO_2. In four animals, one in each of the critically ill groups, the procedure could not be completed due to technical problems: NI/NG ($n=7$), HI/NG ($n=7$), NI/HG ($n=8$), and HI/HG ($n=7$). All data are presented as mean and s.e.m. The gray boxes indicate the mean ± s.e.m of healthy reference ranges ($n=8$). $^{\#}P\leqslant 0.05$ compared with healthy reference ranges. Solid lines: $P\leqslant 0.05$; dashed lines: $0.05<P\leqslant 0.1$ between critically ill groups.

altered oxygen supply, explained the elevated creatinine levels (Table 3b).

Glycolysis and glycogen storage

At day 7 of critical illness, glycogen storage in the renal cortex was increased in the two hyperglycemic rabbit groups compared with the two normoglycemic groups and the healthy reference values (Figure 5). HI/HG rabbits especially had strongly elevated lactate levels in the cortex (Figure 5). These levels correlated positively with cortical glucose content ($R=0.766$; $P<0.0001$). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was lower in hyperglycemic than in normoglycemic critically ill rabbits ($P=0.002$) (Figure 5).

Dicarbonyls as by-products of glycolysis

On day 7, plasma levels of the dicarbonyls glyoxal and methylglyoxal were higher in hyperglycemic than in

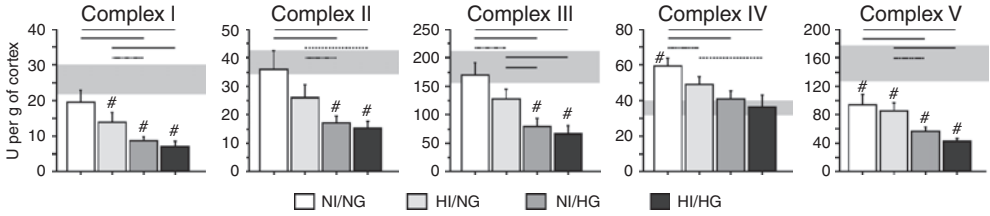

Figure 4 | Mitochondrial respiratory chain enzyme activities. Activities of complexes I, II, III, IV, and V are expressed in U per g wet weight. NI/NG ($n=8$), HI/NG ($n=8$), NI/HG ($n=9$), and HI/HG ($n=8$). HI/HG rabbits had lower protein levels than NI/NG and healthy rabbits. Similar results were obtained for the mitochondrial enzyme activities whether they were expressed per g tissue or per mg of protein (data not shown). Data are presented as mean and s.e.m. The gray boxes indicate the mean ± s.e.m of healthy reference ranges ($n=4$). $^{\#}P\leqslant 0.05$ compared with healthy reference ranges. Solid lines: $P\leqslant 0.05$; dashed lines: $0.05<P\leqslant 0.1$ between critically ill groups.

Table 2 | Correlations of mitochondrial respiratory chain enzyme activities

	Creatinine		Glucose		O_2 to cortex		Dicarbonyls	
	R	*P*-value	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value
Complex I	−0.640	<0.0001	−0.406	0.01	0.423	0.02	−0.531	0.0009
Complex II	−0.729	<0.0001	−0.393	0.02	0.332	0.06	−0.414	0.01
Complex III	−0.686	<0.0001	−0.498	0.002	0.264	0.1	−0.421	0.01
Complex IV	−0.239	0.2	−0.170	0.3	−0.263	0.1	−0.005	>0.9
Complex V	−0.609	<0.0001	−0.517	0.001	0.409	0.02	−0.459	0.005

Table 3 | Stepwise regression analysis weighing impact of glucose, oxygen supply to the cortex, and mitochondrial respiratory chain enzyme activities

Enzyme	Variables in model	Variables not in model	|*R*|	*P*-value
(a) Impact of glucose and oxygen supply on mitochondrial enzyme activities				
Complex I	Glucose, O_2 to cortex	—	0.552	0.005
Complex II	Glucose	O_2 to cortex	0.441	0.01
Complex III	Glucose	O_2 to cortex	0.463	0.008
Complex IV	—	Glucose, O_2 to cortex	—	—
Complex V	Glucose	O_2 to cortex	0.517	0.002
(b) Impact of oxygen supply and mitochondrial enzyme activities on creatinine levels				
Complex I	Complex I	O_2 to cortex	0.551	0.001
Complex II	Complex II	O_2 to cortex	0.605	0.0002
Complex III	Complex III	O_2 to cortex	0.607	0.0002
Complex IV	—	C IV, O_2 to cortex	—	—
Complex V	Complex V	O_2 to cortex	0.528	0.002

Whereas in (a) mitochondrial enzyme complexes in the first column indicate the outcome variable in the analysis, in (b) entries in the first column denote the mitochondrial enzyme that was entered into the model together with oxygen supply to the cortex.

normoglycemic critically ill ($P=0.006$) and healthy rabbits ($P=0.006$) (Figure 6). These levels correlated positively with cortical glucose content ($R=0.679$; $P<0.0001$) and plasma creatinine ($R=0.519$; $P=0.001$) and inversely with mitochondrial respiratory chain enzyme activities (Table 2). The glyoxalase pathway, responsible for detoxification of dicarbonyls, was evaluated by the activity of the glyoxalases and reduced glutathione as a co-factor of glyoxalase I (Figure 6). Glyoxalase I activity was decreased by critical illness, except in NI/NG rabbits. Glyoxalase II activity was significantly reduced only in HI/HG rabbits. Levels of reduced glutathione were lower in HI/HG rabbits than in healthy and normoglycemic sick rabbits.

Effect of hyperglycemia on kidney morphology and mitochondria after 3 days of critical illness

To address the sequence in which mitochondrial abnormalities and morphological kidney injury occurred, we studied the effect of hyperglycemia (HI/NG) compared with that of normoglycemia (HI/HG) on these parameters after 3 days of critical illness. All rabbits survived the preset time course. Mean blood glucose levels were 94.4 ± 6.2 mg per dl in the normoglycemic group and 318.6 ± 6.8 mg per dl in the hyperglycemic group ($P<0.0001$). Creatinine levels were comparable for the two groups at baseline ($P=0.4$), but were higher on day 3 in the hyperglycemic rabbits than in the normoglycemic rabbits (ratio of 1.73 ± 0.10 versus 1.00 ± 0.06, $P=0.0002$). Whereas hyperglycemic rabbits had severe structural kidney injury at day 7, morphology was normal at day 3. In contrast, mitochondrial respiratory chain complexes I and V were affected in the hyperglycemic rabbits already at day 3, although to a lesser extent than on day 7, which was prevented or attenuated by maintaining normoglycemia (Figure 7). Complex I ($R=0.519$, $P=0.047$) and complex V ($R=0.585$, $P=0.02$) activities correlated with mean blood glucose levels. These data suggest that hyperglycemia-induced mitochondrial abnormalities precede structural kidney damage.

DISCUSSION

Our rabbit model of prolonged critical illness showed renal dysfunction, indicated by elevated plasma levels of creatinine. By independent manipulation of blood glucose and insulin levels in these rabbits, we indicated that maintaining normoglycemia during critical illness protected the kidney, whereas elevated insulin levels did not. Kidney protection was not explained by better perfusion or oxygen delivery to the kidney, but rather prevention of hyperglycemia-induced mitochondrial dysfunction may be involved. Indeed, maintenance of normal blood glucose levels prevented cellular glucose overload in the renal cortex, correlating with better preservation of mitochondrial function, which, in turn, correlated with prevention of renal dysfunction.

Blood glucose levels spontaneously increase in our rabbit model of critical illness.[27] Comparable with critically ill patients, these rabbits also develop renal injury as evidenced by elevated plasma creatinine levels in the hyperglycemic

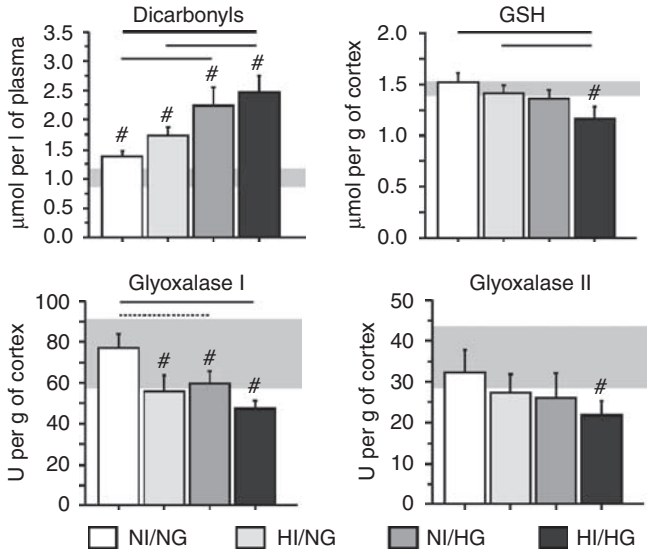

Figure 6 | Metabolism of dicarbonyls. Dicarbonyl levels were measured in plasma. Reduced glutathione levels in the cortex are expressed per g wet weight and enzymatic activities of glyoxalase I and II are expressed in U per g wet weight. NI/NG ($n=8$), HI/NG ($n=8$), NI/HG ($n=9$), and HI/HG ($n=8$). All data are presented as mean and s.e.m. The gray boxes indicate the mean ± s.e.m of healthy reference ranges ($n=4$–8). $^{\#}P\leqslant0.05$ compared with healthy reference ranges. Solid lines: $P\leqslant0.05$; dashed lines: $0.05<P\leqslant0.1$ between critically ill groups.

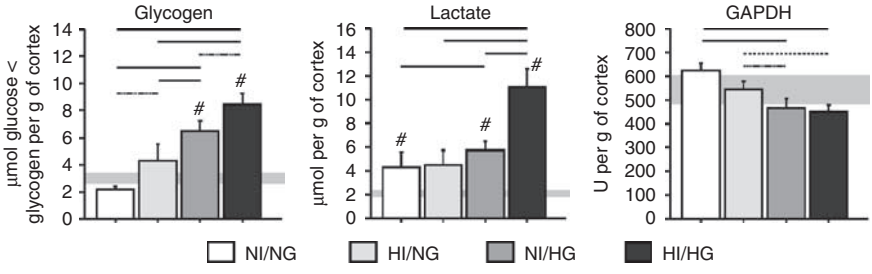

Figure 5 | Evaluation of glucose storage and glycolysis. Glycogen levels are expressed as amount of glucose released from glycogen per g wet weight. Levels of lactate are expressed per g wet weight, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity is expressed in U per g wet weight. NI/NG ($n=8$), HI/NG ($n=8$), NI/HG ($n=9$), and HI/HG ($n=8$). All data are presented as mean and s.e.m. The gray boxes indicate the mean ± s.e.m of healthy reference ranges ($n=4$–8). $^{\#}P\leqslant0.05$ compared with healthy reference ranges. Solid lines: $P\leqslant0.05$; dashed lines: $0.05<P\leqslant0.1$ between critically ill groups.

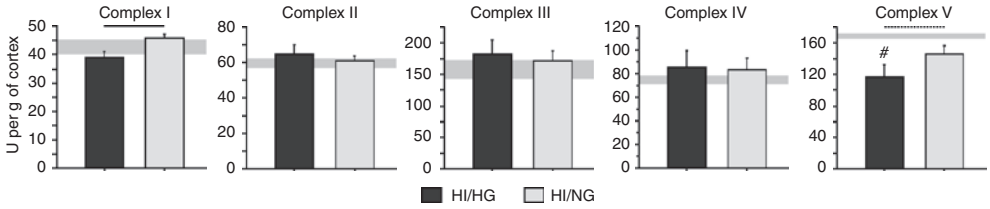

Figure 7 | Mitochondrial respiratory chain enzyme activities after 3 days of critical illness. Activities of complex I, II, III, IV, and V are expressed in U per g wet weight. HI/NG ($n = 5$) and HI/HG ($n = 5$). Data are presented as mean and s.e.m. The gray boxes indicate the mean ± s.e.m. of healthy reference ranges ($n = 5$). $^{\#}P \leqslant 0.05$ compared with healthy reference ranges. Solid line: $P \leqslant 0.05$; dashed line: $0.05 < P \leqslant 0.1$ between critically ill groups.

animals, coinciding with severe morphological abnormalities at the level of the tubuli. Although this condition of AKI is often designated as 'acute tubular necrosis,' frank necrosis appears uncommon.[2,28] Also in our animal model, necrosis was rare. The rabbits, similar to critically ill patients, did not develop glomerular abnormalities. Similar to patients with AKI,[29] the medullary rays appeared particularly susceptible (possibly due to local hypoxia) as with the mildest damage only this region was affected, with progressive involvement of the total cortex (the medullary rays and the convoluted part) with increasing severity. Mild damage was observed in all critically ill rabbit groups. Importantly, the increase in creatinine and severe morphological damage observed in the hyperglycemic ill rabbits was counteracted by maintenance of normal blood glucose levels during critical illness, which protected against cortical glucose overload. Furthermore, a direct correlation was observed between glucose levels in the cortex and plasma creatinine levels. In contrast, hyperinsulinemia was not able to protect the kidney. These data illustrate that blood glucose control, but not insulin by itself, is renoprotective during critical illness.[7–10] The importance of achieving normoglycemia had been suggested earlier by a *post-hoc* analysis of the Leuven clinical studies.[9,10,30] Conclusions regarding a direct effect of insulin or of blood glucose control on kidney could, however, not be drawn, possibly obscured by more severe insulin resistance in the sicker patients as a confounding factor. Indeed, insulin requirement was higher in patients with ICU-acquired RIFLE-Injury or RIFLE-Failure, those with a need for renal replacement therapy, and oliguria.[10]

Despite the high blood flow to and low oxygen extraction by healthy kidneys, these organs are particularly susceptible to hypoxic injury.[31] Hemodynamic alterations resulting in reduced blood flow to the kidney are important in the etiology of AKI.[2,28] Therefore, optimizing the volume status with adequate fluid therapy and cardiac output to assure blood flow and systemic oxygen delivery is of crucial importance, although frequently appears insufficient to prevent renal dysfunction as well as failure of other organs in critically ill patients. Similarly, AKI developed in our animal model, despite adequate fluid resuscitation during the entire study period. The vascular endothelium has a function in regulating microvascular blood flow and oxygen delivery to tissues, and we previously showed endothelial protection by blood glucose control with intensive insulin therapy in patients.[23] We hypothesized that an effect of glucose control or insulin on perfusion and oxygen delivery may have contributed to improved renal outcome in our rabbit model. Critical illness reduced cortical perfusion and oxygen delivery only in the presence of hyperglycemia with concomitant hyperinsulinemia. However, this effect did not explain renal damage.

Renal function strongly correlated with the mitochondrial respiratory chain enzyme activities. These were severely affected by hyperglycemia and protected by strict blood glucose control that also normalized the tissue levels of glucose. Hyperinsulinemia was not directly protective to the mitochondria. A few decades ago, a study on kidneys of third-degree burn-injured rats had shown severely reduced mitochondrial respiration and phosphorylative activity, which could be restored by subcutaneous administration of insulin.[32] However, this study could not discriminate between a role for glycemic control and direct insulin effects. Furthermore, the protection of the kidney and its mitochondria in our study was independent of tissue perfusion and oxygen delivery. Our findings are in agreement with previous studies supporting a role for inadequate mitochondrial oxygen utilization or 'cytopathic hypoxia,' rather than (or in addition to) oxygen delivery, in the altered oxygen metabolism observed in various tissues during critical illness.[33–39] A pivotal role of mitochondrial, bioenergetic abnormalities in renal failure during critical illness is plausible, as the kidney needs a large amount of energy for reabsorption of fluid and solutes across the renal tubular epithelium.[22] Particularly, the proximal tubule appears very sensitive to mitochondrial injury. The mitochondrial abnormalities in the critically ill rabbits did not develop secondary to major structural injury, as mitochondrial respiratory chain activity reductions preceded structural alterations, further supporting the involvement of mitochondrial damage in the pathophysiology of hyperglycemia-induced kidney injury.

The mechanisms underlying rapid glucose toxicity to mitochondria and kidney in critical illness remain incompletely understood. A number of pathways are known to be activated in the hyperglycemic milieu and have been suggested to contribute to hyperglycemic damage in diabetes mellitus. One of these pathways involves dicarbonyls,

which are spontaneously formed as metabolites of glucose and are highly reactive with proteins.[40] Elevated dicarbonyl levels are observed in diabetes,[41] mimic diabetic complications when administered in the presence of normoglycemia,[42] and act as mitochondrial toxins in the kidney or other tissues.[43–46] In the kidney of a diabetes rat model, specific mitochondrial proteins appeared post-translationally modified by dicarbonyls, of which the extent correlated with progressively compromised mitochondrial bioenergetics.[46] The critically ill hyperglycemic rabbits in our study had elevated dicarbonyl levels compared with normoglycemic rabbits, which correlated with mitochondrial damage and renal dysfunction and hence may point to a contribution to the pathogenesis of kidney damage in critical illness. Dicarbonyls may accumulate secondary to inactivation of the glycolytic enzyme, GAPDH, by hyperglycemia that blocks glycolysis and shifts glucose into proximal toxic pathways[47] or, alternatively, by increased glucose flux through the glycolytic pathway that proportionally increases its diversion into these toxic pathways.[48] A small decrease was observed in the activity of GAPDH in the cortex of hyperglycemic rabbits. However, the parallel increase in the tissue levels of lactate, strongly correlating with glucose content, suggests that glycolytic flux was not compromised, but rather increased. Such increased formation of lactate due to increased glucose turnover and higher production of pyruvate has previously been shown in critically ill patients,[49,50] despite the fact that a classical dogma links hyperlactatemia in critical illness to inadequate tissue oxygenation or to impaired clearance.

Although our burn-injured rabbit model of critical illness mimics the human stress response to severe trauma,[51] inherent limitations need to be considered when working with animal models in view of extrapolation to patients and generalizability of the findings. Without active glucose control, burn injury in our animal model spontaneously results in the degree of hyperglycemia in this study.[27] However, although such high glucose levels are still observed in certain patient populations and used in experimental studies,[52–54] they are not currently tolerated in critically ill patients in general. Avoiding excessive hyperglycemia may be key to obtain renal and other clinical benefits of intensive insulin therapy, as observed in the Leuven studies,[7–10] and suggested by the present animal data. Whether blood glucose should be controlled to strict normoglycemia, as done in the Leuven studies, or rather to an intermediate target range should be further studied using appropriate glucose-monitoring tools.[7–21] A certain bias may have been introduced to our animal study by arbitrarily choosing a 7-day time course of critical illness, in view of the potential dynamic character of the changes and the study of survivors only (to avoid interference by post-mortem artifacts), that excluded the most severely ill animals from the analyses. However, such selection due to the higher mortality of hyperglycemic animals would underestimate rather than overestimate the observed differences between the normoglycemic and hyperglycemic rabbit groups. Furthermore, we have largely circumvented these limitations by also analyzing a time course of 3 days of critical illness, already showing discrete mitochondrial alterations early in the disease, in the absence of mortality as a confounding factor. Owing to technical reasons, urine could not be collected during the study, hampering further characterization of the renal dysfunction (e.g., by determining urine output and calculating glomerular filtration rate). However, qualitative deterioration of glomerular filtration rate could be evaluated by change in creatinine levels in the course of the disease.[1] Finally, analyses were inevitably performed on small pieces of tissue, which theoretically may have detected regional differences rather than alterations at the whole organ level.

In conclusion, hyperglycemia evoked cellular glucose overload in the kidney of critically ill rabbits and induced mitochondrial dysfunction and renal injury. Maintenance of normoglycemia, independent of insulinemia, protected against such a cascade of damage to the kidney.

MATERIALS AND METHODS

Experimental rabbit study

This study was largely performed on plasma and tissue samples obtained during a previously reported experimental study[25] in a well-characterized standardized, fluid-resuscitated, third-degree burn injury model of critical illness. The study was approved by the Leuven University Ethical Review Board for Animal Research (P04058). All animals were treated according to the *Principals of Laboratory Animal Care* formulated by the US National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the US National Institutes of Health.

The study protocol has been described in detail elsewhere.[25] In adult, 3- to 4-month-old male New Zealand white rabbits weighing ~3 kg, endogenous insulin production was eliminated with alloxan (150 mg/kg injected slowly by a marginal ear vein on day −1). At day 0, catheters were placed in the right jugular vein for intravenous infusion of insulin/total parenteral nutrition and in the right carotid artery for blood sampling, followed by application of a full thickness 20% body surface area third-degree burn injury, all under general anesthesia (30 mg/kg ketamine, intramuscularly, 0.15 ml/kg medetomidine intramuscularly, 1.5% isoflurane inhalation). A paravertebral block was performed with 5 ml 1% xylocaine before burn injury. These critically ill rabbits were randomized to glucose control to normal (80–110 mg per dl, NG) or high levels (250–350 mg per dl, HG). For each glucose level, insulin was clamped to normal (1.2 U/kg/day infused, NI) or high levels (4 U/kg/day infused, HI): NI/NG ($n=8$), HI/NG ($n=8$), NI/HG ($n=9$), and HI/HG ($n=8$). Animals were fluid resuscitated by infusion of the 18 ml/h Hartmann solution supplemented with 3.4% glucose. During the experimental period, the two preset levels of glycemia were reached by adjusting a continuous 50% glucose infusion supplementing basal glucose intake. At day 1, the Hartmann solution was replaced by parenteral nutrition infused at 12 ml/h, which was changed daily until day 7. The amount of nutrition given was recorded daily. The study was continued until in each group eight animals survived to day 7. Animals were fluid resuscitated throughout the 7-day study period.

On day 7, animals were anesthetized and normoventilated through tracheostomy. Normovolemia was maintained by infusion of 6 ml/h of Hartmann solution. The carotid artery catheter (previously placed for blood sampling) was advanced into the left ventricle, guided by the pressure curve. Hemoglobin, pO_2 (paO_2), pCO_2, and oxygen saturation (SaO_2) were determined in arterial blood samples and cardiac output was recorded as described.[25] Arterial oxygen content CaO_2 (hemoglobin $\times 1.36 \times SaO_2 + paO_2 \times 0.0031$) and delivery DaO_2 (cardiac output $\times CaO_2$) were calculated. Perfusion of the kidneys was evaluated using colored Dye-Trak microspheres according to a previously published protocol (Triton Technology, San Diego, CA, USA).[55] To this end, a fluid-filled catheter was inserted 3 cm deep through the carotic artery into the circle of Willis. The catheter was connected to a glass syringe mounted in a precision pump (Harvard Pump 11, Harvard Apparatus, Holliston, MA, USA) for withdraw of a reference sample. The withdraw pump was started at 1 ml/min, and 10 s later, 3 million microspheres with highly uniform size distribution (15.00 ± 0.45 μm) in a 1-ml volume were injected over 10 s through the left ventricular catheter. The line was then flushed for 40 s with Hartmann solution, and 60 s later, the pump stopped yielding a reference sample at 120 s, which was stored at 4 °C until analysis. After this procedure, slices of kidney and biopsies from the renal cortex were taken and snap-frozen in liquid nitrogen for long-term storage or stored at 4 °C for recovery of microspheres. Animals were killed by cutting out the heart.

Microspheres were recovered from the tissue and blood samples by KOH digestion and filtration through 8 μm filters.[55] The dye was extracted from the microspheres in 100 μl dimethylformamide and its concentration determined in a spectrophotometer.[55] Recovery of microspheres from the reference sample was used to calculate absolute organ blood flow. Equal perfusion of the left and right kidney was used as a quality control. Oxygen supply was calculated as the product of perfusion estimated by the microspheres and CaO_2.

As a reference, data and tissue biopsies from healthy rabbits were obtained and stored similarly as for the critically ill rabbits. These rabbits did not receive alloxan and burn injury, were not catheterized, and had free access to standard chow.

To assess early effects of hyperglycemia, we designed a second animal study, where we created two groups relevant to the human condition and killed the animals after 3 days of critical illness. During the 3-day time course, the rabbits were controlled to either normoglycemia or hyperglycemia with concomitant hyperinsulinemia (HI/NG, $n = 5$ and HI/HG, $n = 5$, respectively), to mimick the clinical condition of critically ill patients (elevated insulin levels regardless of insulin therapy[56]). Slices of kidney and biopsies from the renal cortex of these ill as well as healthy rabbits ($n = 5$) were taken and stored at −80 °C as described.

Biochemical analyses

Plasma creatinine was measured on day 7 by routine clinical chemistry (Jaffé method). Renal structure was investigated by light microscopy after periodic acid-Schiff staining of paraffin sections. Activities of the mitochondrial respiratory chain enzyme complexes I–V were measured as described.[24] The levels of glucose, lactate, glycogen, and glutathione[57,58] and the activities of GAPDH and glyoxalase I and II[24,59] were quantified by spectrophotometric assays. Dicarbonyls (glyoxal and methylglyoxal) were quantified by mass spectrometry.[60] All analyses were carried out blinded to treatment.

Statistical analysis

Results are presented as mean and s.e.m. Differences among study groups were analyzed by multifactorial ANOVA and Fisher's protected least-significant difference *post-hoc* testing for comparison of normally distributed data and by the Kruskall–Wallis and Mann–Whitney *U*-test for not-normally distributed data. Correlations between parameters were studied by linear, logarithmic, or stepwise regression analysis. Differences were considered statistically significant when two-sided *P*-values were $\leqslant 0.05$. Statistical analyses were carried out using StatView 5.0.1 for Macintosh (SAS Institute, Cary, NC, USA).

DISCLOSURE

All the authors declared no competing interests.

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