Hemorrhagic shock and protein O-linked β-N-acetylglucosamine modification

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ABBREVIATIONS

ATP: adenosine triphosphate

ADP: adenosine diphosphate

ALT: alanine aminotransferase

AST: aspartate aminotransferase

BSA: bovine serum albumin

BUN: blood urea nitrogen

CTD 110.6: C-terminal domain

cTnI: cardiac troponin I

DRP: drag reducing polymer

eNOS: endothelial nitric oxide synthase

ER: endoplasmic reticulum

GalNAc: N-acetylgalactosamine

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GFAT: glutamine: fructose-6-phosphate amidotransferase

GlcN: glucosamine-hydrochloride

GlcNAc: N-acetylglucosamine

Glc-6-P: glucose-6-phosphate

GP: glycogen phosphorylase

GRP78: glucose regulated protein

GSH: reduced glutathione

H_2O_2: hydrogen peroxide

HBP: hexosamine biosynthesis pathway

HES: hydroxyethyl-strach

HSP: heat shock protein

ICAM: intercellular adhesion molecule

iNOS: inducible nitric oxide synthase

IL: interleukin

LDH: lactate dehydrogenase

LVDP: left ventricular developed pressure

MAP: mean arterial pressure

MODS: multiple organ dysfunction syndrome

MPO: myeloperoxidase

NADPH: nicotinamide adenine dinucleotide phosphate

NAG-thiazoline: 1,2 dideoxy-2`-methyl-α-D-glucopyranoso(2,1-d)-Δ2`-thiazoline

NF-κB: nuclear factor kappa-B

OGN: β-N-acetylglycosidase/O-GlcNAcase

O-GlcNAc: O-linked β-N-acetylglucosamine

OGT: O-GlcNAc transferase

PAGE: polyacrylamide gel electrophoresis

PCA: perchloric acid

PUGNAc: O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate)

ROS: reactive oxygen species

SIRS: systemic inflammatory response syndrome

SDS: sodium dodecyl sulfate

TH-R: trauma-hemorrhage and resuscitation

TNF: tumor necrosis factor

TTO4: 2[(4-chlorophenyl)imino]tetrahydro-4-oxo-3-[2-tricyclo(3.3.1.13.7)dec-1-ylethel]

UDP-GlcNAc: uridine diphospho-N-acetyl-glucosamine

UDP-GalNAc: uridine diphospho-N-acetyl-galactosamine

UDP-HexNAc: uridine diphospho-N-acetyl-hexosamine

INTRODUCTION

Trauma and hemorrhagic shock

Despite the advances in traffic and occupational safety, severe injuries are among the leading causes of death in both civilian and combat environment. Importantly, trauma remains the major cause of death in people younger than 35 years. Hypovolemia due to hemorrhage is a major contributor in nearly half of 150.000 deaths per year in the United States, attributed to traumatic injury. Importantly, in numerical terms, injuries claim the lives of 235.000 European Union citizens annually, the intent being reported accidental deaths in two-thirds of the event.

The early treatment of hemorrhagic shock includes surgical intervention and restoration of the intravascular volume by means of crystalloid or colloid infusion and blood transfusion. However, a massive resuscitation strategy before the surgical control of the ongoing bleeding may result in further blood loss and higher mortality rate. Therefore, there is an emerging interest in small or minimal-volume resuscitation strategies, especially in rural civilian areas or in combat environments, where the prompt surgical intervention is not available, due to significant delays in transport.

Severe injuries with significant blood loss are typically associated with hypovolemic shock causing decreased tissue perfusion with upset balance between oxygen demand and supply. Hemorrhagic shock is also related with the release of pro-inflammatory mediators and free radicals. An excessive pro-inflammatory response, called Systemic Inflammatory Response Syndrome (SIRS) is an important factor in the development of Multiple Organ Dysfunction Syndrome (MODS). This complex organ failure occurs with a relatively high incidence (11.4%) and associated with high mortality (61%).

Accordingly, early attenuation of tissue injury combined with down-regulation of pro-inflammatory mediators could significantly decrease the trauma-related mortality. Unfortunately, beside the well established therapeutic protocols, i.e. prompt surgical intervention and supportive intensive therapy care; there is only a few, effective metabolic intervention has been successful in multiple, prospective human trials, despite of the encouraging results of animal experiments. ***Therefore, there is a need for new metabolic treatments to improve both early survival and the later outcome of severely injured patients.***

Trauma and stress-induced hyperglycemia

Acute hyperglycemia is frequently associated with injuries, such as trauma and hemorrhagic shock. Stress induced hyperglycemia has been reported to be an adaptive mechanism and prevention of this hyperglycemic response in rats by food-deprivation increased mortality following hemorrhage. On the other hand, in the recent clinical practice, the early euglycemic control has been proven to improve the outcome of traumatized and intensive care unit (ICU) patients.

Notably, high glucose levels facilitate the flux through the hexosamine biosynthesis pathway (HBP), which has been linked, among others, to improved cell survival under stress-conditions.

Hexosamine Biosynthesis Pathway (HBP) and protein O-GlcNAcylation:

It is estimated from *in vitro* cell culture studies that between 2% and 5% of total glucose entering the cell is metabolized via the hexosamine biosynthesis pathway (HBP; Fig. 1). Glucose entry into the HBP is regulated by L-glutamine-D-fructose 6-phosphate amidotransferase (GFAT), which converts fructose-6-phosphate to glucosamine-6-phosphate with glutamine as the amine donor. Glucosamine-6-phosphate is then metabolized via various hexosamine intermediates, leading to the synthesis of UDP-GlcNAc. UDP-GlcNAc provides glycosidic precursors for the synthesis of glycoproteins, glycolipids, and proteoglycans; it is also the essential sugar nucleotide donor for the formation of O-GlcNAc-modified proteins.

The role of O-GlcNAc in regulating cellular function have been linked to various diseases, such as insulin resistance and the adverse effects of diabetes and the development of cancer and neurodegenerative disorders such as Alzheimer disease. However, in contrast to these adverse effects, Zachara et al. showed that in mammalian cells, stress stimuli increased protein O-GlcNAc levels, and augmentation of this response increased tolerance to the same stress stimuli.

Flux through the HBP can be increased with the addition of exogenous glucosamine, which enters cells via the glucose transporter system and is phosphorylated to glucosamine-6-phosphate by hexokinase, thus bypassing the rate-limiting enzyme GFAT and leading to a rapid increase in UDP-GlcNAc levels.

Regulation of protein O-GlcNAcylation

The attachment of a single β-N-acetylglucosamine moiety via an *O*-linkage to specific serine/threonine residues of nuclear and cytoplasmic proteins is catalyzed by O-GlcNAc transferase, (OGT) using UDP-GlcNAc as the obligatory substrate. The global extent of O-GlcNAc modification has been reported to be tightly dependent on the flux through HBP, since OGT catalytic activity is highly sensitive to changes in UDP-GlcNAc concentrations. The level of O-GlcNAc on nuclear and cytoplasmic proteins is also regulated by β-N-acetylglucosaminidase (O-GlcNAcase, OGN), which catalyzes the removal of the sugar moiety from proteins. Both OGT and OGN are highly conserved in mammals. In contrast to OGT, which is reported to be localized primarily to the nucleus, the active form of O-GlcNAcase is predominantly localized to the cytoplasm (90%). Interestingly, recent studies provide support for the concept of a complex and reciprocal relationship between phosphorylation and O-GlcNAcylation.

Pharmacological studies of the rate of O-GlcNAc formation and removal have been limited due to the lack of specific high-affinity OGT inhibitors. Therefore, the most widely used pharmacological approach to modulate O-GlcNAc levels has been O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), an analog of GlcNAc that is an effective and relatively specific competitive inhibitor of O-GlcNAcase. By inhibiting O-GlcNAcase, PUGNAc slows or prevents the removal of O-GlcNAc, leading to a relatively rapid increase in O-GlcNAc levels. The effectiveness of PUGNAc in increasing O-GlcNAc has been demonstrated in a variety of biological systems. Recently, new O-GlcNAcase inhibitors (NAG-thiazoline, GlcNAcstatin) have been introduced and found to be more effective and more selective than PUGNAc.

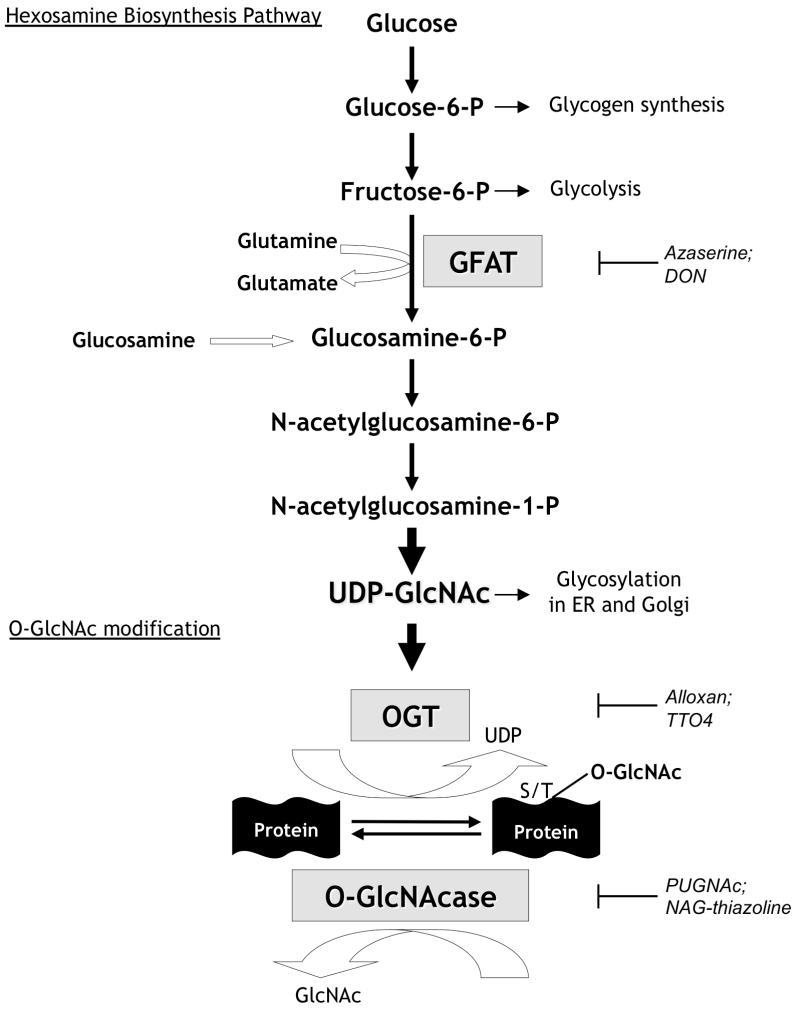

Fig.1. The hexosamine biosynthesis pathway (HBP) and protein O-GlcNAcylation. */Adapted from Laczy B et al., AJP Heart Circ Physiol 296:13-28, 2009/ glucose-6-phosphate (glucose-6-P), fructose-6-phosphate (fructose-6-P), glucosamine-6-phosphate, L-glutamine-D-fructose 6-phosphate amidotransferase (GFAT), UDP-N-acetylglucosamine (UDP-GlcNAc), 6-diazo-5-oxo-L-norleucine (DON), O-diazoacetyl-L-serine (azaserine), endoplasmic reticulum (ER), uridine-diphospho-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyltransferase (OGT), O-linked β-N-acetylglucosamine (O-GlcNAc), β-N-acetylglucosaminidase (O-GlcNAcase), uridine analog: alloxan 2[(4-chlorophenyl)imino]tetrahydro-4-oxo-3-[2-tricyclo(3.3.1.13.7)dec-1-ylethel] (TTO4), O-(2-acetamido-2'-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc), 1,2 dideoxy-2'-methyl-D-glucopyranoso(2,1-d)-2'-thiazoline (NAG-thiazoline). S/T, serine/threonine.*

Protein O-GlcNAcylation and ischemia-reperfusion injury

A growing body of data demonstrates that activation of pathways leading to increased O-GlcNAc formation enhances tolerance to stress and improves cell survival. In neonatal cardiomyocytes, hypoxia-reoxygenation causes a transient increase in O-GlcNAc levels, and augmentation of this response by elevating flux through HBP with high glucose, glucosamine, or PUGNAc improves cell viability and decreases necrosis and apoptosis. In the isolated perfused heart, activation of the HBP with either glucosamine or glutamine before the induction of ischemia significantly increased cardiac O-GlcNAc levels, improved contractile

function, and decreased tissue injury after reperfusion. Furthermore, Laczy B et al. recently proved that inhibition of OGN in perfused rat hearts during reperfusion by NAG-thiazolines is cardioprotective in an O-GlcNAc dependent manner. Importantly, they found that inhibiting OGA might be a clinically relevant approach for ischemic cardioprotection, in part, by preserving the integrity of O-GlcNAc- associated Z-line protein structures. *In vivo* administration of PUGNAc has been shown to reduce infarct size after ischemia-reperfusion in mice.

Several putative mechanisms have been put forward to explain the increased tolerance to stress associated with increased O-GlcNAc levels. Increasing O-GlcNAc levels have been associated with increased transcription of HSP40 and HSP70 levels, and the latter is a known target for O-GlcNAc modification. Preliminary studies have also shown that ischemia-reperfusion alters the level of O-GlcNAc modification of glycogen phosphorylase b, mitochondrial aconitase 2, and the cytoskeletal protein vinculin. Increased levels of O-GlcNAc have also been reported to inhibit protein degradation most likely due to inhibition of the proteasome and this could contribute to enhanced cell survival. It is also possible that cardioprotection is mediated via inhibition of Ca^{2+}-overload on reperfusion.

These results provide strong evidence that the protective effect seen associated with increasing O-GlcNAc levels at the cellular and isolated organ levels can be translated to the in vivo environment.

Protein O-GlcNAcylation, hemorrhagic shock and inflammation

Rodent trauma-hemorrhage models are widely used in an effort to reproduce a real life situation of severely injured patients with significant blood loss. These models are generally based on combination of soft tissue trauma and blood withdrawal, followed by fluid (or blood) resuscitation. The pathomechanism of damage caused by trauma-hemorrhage models is basically a general severe ischemia-reperfusion insult. It has been demonstrated that administration of glucosamine or PUGNAc midway resuscitation significantly improved cardiac output and increased perfusion of critical organs systems compared with vehicle-treated controls 2 hours after trauma-hemorrhage. *In vivo* administration of glucosamine or PUGNAc also increased O-GlcNAc protein levels in multiple tissues; supporting the notion that enhanced O-GlcNAc levels on nucleocytoplasmic proteins mediated the protection.

A number of studies have demonstrated that acute increases in O-GlcNAc attenuate the inflammatory response induced by tissue injury and stress. Acute activation of O-GlcNAc formation has been shown to be protective in the setting of endoluminal vascular injury in vivo. Pretreatment with either glucosamine or PUGNAc increased O-GlcNAc-modified protein levels in balloon-injured rat carotid arteries compared with vehicle-treated controls. In isolated cardiomyocytes, both glucosamine and OGT overexpression increase O-GlcNAc levels, attenuate LPS-induced TNF-α and ICAM-1 expression, and decrease IκB-α phosphorylation and nuclear NF-κB levels. We have recently demonstrated that O-GlcNAcase inhibitor PUGNAc treatment significantly decreased serum and BAL (bronchoalveolar lavage) levels of IL-6 and attenuated ICAM-1 expression in the lung after CLP (cecal puncture and ligation)-induced sepsis in rats. The PUGNAc mediated decrease in CLP-induced inflammation was associated with a ~2-fold increase in O-GlcNAc levels.

These findings further support our hypotheses that increased O-GlcNAc levels could improve survival, decrease organ injury and inflammation after severe hemorrhagic shock.

AIMS AND HYPOTHESES

Protein O-GlcNAcylation is a post-translational modification and it has been linked to improved cell survival following ischemia-reperfusion injuries. Severe injuries are one of the leading causes of death and there is a need for new metabolic treatments to improve both survival and the later outcome of severely injured patients. Therefore, in our study, we pursued the following aims:

» To examine the effect increased O-GlcNAc levels on the short-term outcome after severe trauma-hemorrhage in rats

- The goal of the first part of the study was to demonstrate whether a small bolus intravenous administration of glucosamine improves the survival rate, effects hemodynamic parameters and tissue energetic status after severe trauma-hemorrhage in the absence of large volume resuscitation.

- We also examined whether these changes are associated with a significant increase in the protein O-GlcNAc levels in the heart, brain, and liver extracts.

» To examine the effect of increased proteins O-GlcNAc levels on later outcome after severe trauma-hemorrhage in rats

- The purpose of the second part of the study was to determine whether increasing O-GlcNAc levels with either glucosamine or PUGNAc improves 24-hr survival in rats following severe trauma-hemorrhage.

- It was also evaluated whether the improved survival was associated with decreased organ injury, apoptosis and attenuation of pro-inflammatory responses.

MATERIALS AND METHODS

Short-term (2 hours) survival model of trauma-hemorrhage: Here we used a volume-controlled hemorrhage model, that in the absence of any intervention exhibited 0% survival within 35 min after the end of the hemorrhage period. Briefly, under isoflurane anesthesia (1.5% vol/vol, 1 L/min oxygen flow), two femoral arteries and the right femoral vein were cannulated with for continuous blood pressure monitoring blood withdrawal and drug administration. A 5-cm-long midline laparotomy was performed to induce a soft tissue damage to mimic trauma prior to hemorrhage. Animals were kept on heating pad with temperature set to 37 ºC in effort to maintain central body temperature, Hemorrhage was induced by withdrawal of 55% of the calculated total blood volume (total blood volume [mL] = body weight [g] × 0.061) (80) for 25 min using a syringe pump (Harvard Instruments, Holliston, MA). ***Experimental groups:*** At the end of the hemorrhage, the rats were divided randomly into two groups: glucosamine- and mannitol-treated (control) animals. Due to the relatively high osmolarity of glucosamine solution, the mannitol served as an osmotic control. In the glucosamine group, 2.5 mL of 150 mM glucosamine solution was administered i.v. for 10 min. The animals were observed for 2 h after treatment or until death (apnea duration > 1 min). At the end of the experiment, the surviving rats were euthanized by means of i.v. injection of concentrated potassium chloride solution. Then, different blood and organ samples were collected.

Long-term (24 hours) survival model of trauma-hemorrhage: We adapted our severe, volume-controlled hemorrhagic shock model, described above, by providing full resuscitation after blood withdrawal and 45 min of pressure-controlled hypovolemia. Briefly, hemorrhage was induced by withdrawal of 55% of the calculated total blood volume for 25 min. Then, mean arterial pressure was maintained on 35-40 mmHg for 45 minutes (pressure controlled phase) by i.v. administration of small volumes of 0.9% NaCl (i.e., normal saline) solution until a maximum of 40% of the shed blood volume was returned. This was followed by resuscitation, with four times of total withdrawn blood volume of i.v. 0.9% NaCl, administered over 60 minutes. The catheters were removed, animals were allowed to wake up and observed up to 24 hours after resuscitation, at which point surviving animals were euthanized by i.v. injection of concentrated KCl solution. Blood, heart, lung and liver were collected for subsequent analyses as described below. During the 24 hours observation period any animals that were found to be moribund were euthanized and considered dead with respect to the survival analysis. ***Experimental groups****:* Animals were randomly assigned to the following 4 groups: 1) Sham surgery; 2) Control (untreated) trauma-hemorrhage; 3) trauma-hemorrhage with glucosamine treatment (270 mg/Kg body weight) and 4) trauma-hemorrhage with PUGNAc treatment (7 mg/Kg body weight). In both treatment groups, 25% of total dose in a 2 mL increment was administered immediately after hemorrhage and the remaining 75% continuously during resuscitation. Sham surgery animals underwent only general anesthesia and vessel cannulation. All animals received 0.3 mg/Kg b.w. buprenorphine subcutaneously immediately following and 12 hours after resuscitation.

Blood gas analysis, serum enzyme, glucose and electrolyte measurements: Blood gas parameters, electrolytes, glucose and lactate levels from arterial samples were analyzed by

using commercially available blood gas analyzer. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), amylase, creatinine and blood urea nitrogen (BUN) were measured using enzymatic reaction assays.

Serum cytokine levels: Serum levels of interleukin- (IL) 6 and 10; and tumor necrosis factor (TNF)-α were measured with commercially available sandwich enzyme-linked immunosorbent assay kits, following the manufacturer's guidelines.

Western-immunoblots analyses: Proteins were separated on a 7-10 % SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) electrophoresis gel and transferred either to polyvinylidene difluoride or pure nitrocellulose membranes. Protein loading was confirmed by Sypro Ruby (Bio-Rad) staining. Membranes were incubated overnight with 1:1000 or 1:2500 dilution of CTD 110.6 (anti-O-GlcNAc), phosphoserine, phosphothreonine, GRP78 or iNOS. For loading control, membranes were stripped and blots were incubated with β-actin or calsequestrine antibody. The densitometry of CTD 110.6 and phosphoserine / threonine immunoblots were performed by selecting the whole lane (all bands) in case of each sample and measuring the mean density of selected areas after background subtraction, using Scion Image analysis software (Scion Corporation, Frederick, MD).

High-performance liquid chromatography measurements (HPLC): Tissue adenosine triphosphate (ATP) levels: frozen tissue powder was homogenized in 0.3M perchloric acid, centrifuged for 10 min under a temperature of 4 ˚C at 14,000 g; then, the supernatant was mixed with 1:4 trioctylamine:1,1,2-trichlorotrifluoroethane. The mixture was centrifuged for 5 min under a temperature of 4 ˚C; then, the aqueous phase was loaded onto a strong anion exchange column with 262-nm wavelength of detection. Serum glucosamine levels: 0.1 mL of serum was added to 0.4 mL acetonitrile to precipitate the serum proteins. The purified and dried samples were derivatized with 0.2 mL of 88 mg/mL 1-naphthyl-isothiocyanate; then, the reaction was stopped by using 0.4 mL of 1.5% acetic acid. The excess derivatizing reagent was partitioned into an organic phase by the addition of 1.0 mL of chloroform. The aqueous layer was purified by means of an anion exchange cartridge and the eluted solution was loaded onto ODS2 analytical column. The samples were run with an isocratic system. Data were analyzed and quantified as area under the curve by using the System Gold Nouveau software (Beckman Coulter, Fullerton, CA).

Nuclear factor kappa-B (NF-κB), Apoptosis and Myeloperoxidase (MPO) assays: NF-κB was measured with an ELISA-based, colorimetric, oligonucleotid-binding assay. Apoptosis was determined using special ELISA kit measuring degraded DNA-associated histones. MPO levels were also determined using commercially available ELISA kits.

Data Analysis: Statistical analyses were performed, using GraphPad Prism 4 software (GraphPad, San Diego, CA) and SPSS13.0 (SPSS, Chicago, IL). The survival data were compared, using log-rank test, and were presented as survival percentage. Other statistical comparisons were performed, using unpaired Student's t test, correlation or parametric one-way analysis of variance with Bonferroni's or Dunnett's post hoc test, as appropriate. Data that were not normally distributed and/or of unequal variance underwent either log or rank transformations, and the analyses were performed on the transformed data with one-way or Kruskal-Wallis analyses of variances with Dunn's post hoc test, respectively.

RESULTS

I. The effect of increased protein O-GlcNAc levels on the short-term outcome after severe trauma-hemorrhage in rats

Survival rate

A total of 37 animals were subjected to trauma-hemorrhage with minimal resuscitation. Glucosamine significantly improved the survival, compared to the (mannitol-treated) Control animals (survival rate after 2 h, 47% vs. 20%, respectively; $p<0.05$) Fig. 2.

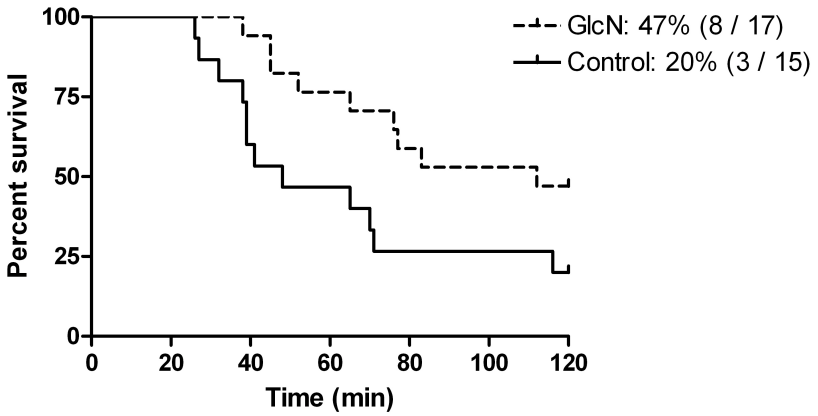

Fig. 2. Percent survival over time for Glucosamine-treated (GlcN) and Control (mannitol-treated) rats *(p<0.05, log-rank test). The survival percentage, the number of survival animals and the total number of animals subjected to TH in each groups are indicated on figure legend. Zero (0) minute indicates the start of the drug administration.*

Hemodynamic parameters

The mean arterial pressure was significantly higher for 18 min after treatment in the glucosamine group. Similar results were observed when comparing only those animals in the groups of animals that survived the 2-h follow-up period.

Arterial blood gas, serum biochemical parameters and serum glucosamine levels

The P_{ACO_2}, acid base status (pH), base excess (BE), and serum bicarbonate decreased in both groups after trauma-hemorrhage compared with the baseline, indicating the severity of the shock. There was also a marked increase in serum lactate levels, consistent with the development of severe metabolic acidosis. Hematocrit was also significantly decreased by the end of the hemorrhage and showed a further decrease 30 min after treatment. The serum potassium and phosphate levels were significantly increased at the end of the hemorrhage in both groups, parallel with the severity of shock.

However, we found no significant differences comparing serum enzyme levels (ALT, AST, LDH, amylase, creatinine and blood urea nitrogen) at any time point. Despite the effect

of glucosamine on survival rates, there were no significant differences between the glucosamine- and the mannitol-treated Control group in any of these parameters.

Thirty minutes after treatment with glucosamine, the serum glucosamine concentration was 2.6 mM ± 0.5 mM. In the Control group and in the Nonoperated control group, the serum glucosamine levels were undetectable.

Tissue energetic state

Hemorrhagic shock had no effect on ATP levels in heart, brain, or abdominal muscle; however, there was a marked decrease in ATP levels in liver. The tissue ATP levels were not altered by glucosamine treatment in any organ.

Serum cytokines

The serum levels determined 30 min after the treatment of TNF-α and IL-10 were slightly increased compared with Nonoperated rats; this finding was not altered by the glucosamine treatment. The serum IL-6 levels remained undetectable during the experiment.

Protein O-GlcNAc levels

Initially, the tissue samples were harvested 2 h after the end of hemorrhage or when the death of the animal was confirmed; however, the comparison of the tissue O-GlcNAc levels at different time points did not show significant differences between the groups.

Therefore, additional animals were subjected to the same trauma-hemorrhage procedure followed by mannitol or glucosamine treatment. The different organ samples were collected 30 min after treatment, shortly after the period when the increase in MAP was significantly higher in the glucosamine-treated group.

Glucosamine treatment significantly increased the protein O-GlcNAc in heart (Fig. 3), brain, and liver samples compared with the Control animals; however, the O-GlcNAc levels in abdominal muscle were not significantly increased.

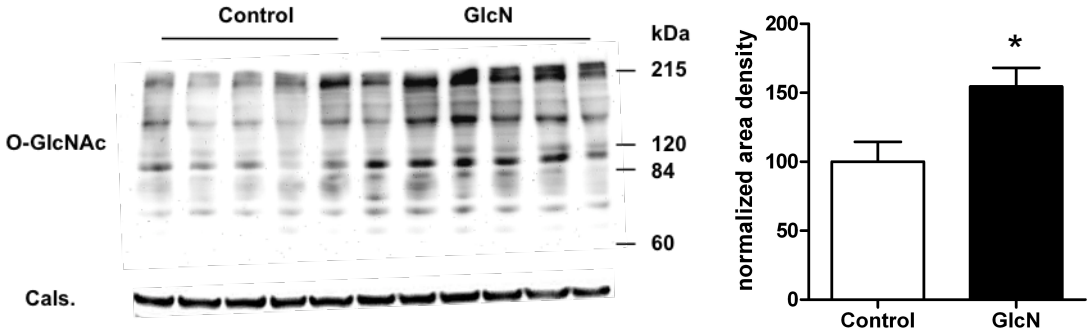

Fig. 3. O-GlcNAc immunoblot of the Heart from GlcN (glucosamine-treated) and Control (mannitol-treated) rats 30 min after TH and drug administration *is shown on the left side. Bar chart on the right side represenst the mean of related area densities ± SEM. Mean densities were normalized to calsequestrin (Cals) staining. *p ≤ 0.05 Vs Control.*

II. The effect of increased proteins O-GlcNAc levels on later outcome after severe trauma-hemorrhage in rats

Survival rate

A total of 50 animals were subjected to the trauma-hemorrhage and full resuscitation protocol (TH-R) and observed for 24 hours. Four animals died during either the initial surgical procedure or early during the resuscitation phase and were therefore excluded from any subsequent analyses. The survival rate was significantly increased by both glucosamine (85%) and PUGNAc (86%) treatment, compared to the Control animals (53%) (Fig. 4). No mortality was observed in the Sham surgery group (data not shown).

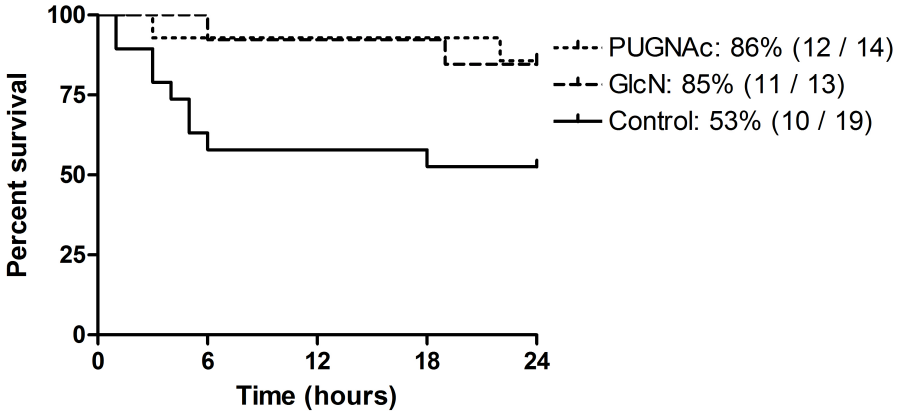

Fig. 4. Survival curve: Percent survival over time for Control, GlcN and PUGNAc treated rats. *The survival percentage, the number of survival animals and the total number of animals subjected to TH-R in each groups are indicated on figure legend. Both GlcN and PUGNAc treatment significantly improved survival, compared to Controls (Log-rank test, p<0.05). Zero (0) hour indicates the end of resuscitation.*

Arterial blood gas and serum biochemical parameters

Hemorrhagic shock induced hyperkalaemia, hypocalcaemia as well as severe acidosis, as indicated by decreased partial pressure of carbon dioxide (PCO_2), pH, bicarbonate (HCO_3^-), total carbon dioxide (TCO_2), base excess (BE) and increased serum lactate levels. Following blood withdrawal, the acidosis was similar in all groups; however, by the end of resuscitation, it was significantly improved by both GlcN and PUGNAc administration, compared to Control animals, as indicated by increased HCO_3^-, TCO_2, BE and decreased serum lactate levels.

Serum cytokines

Hemorrhagic shock induced a significant increase in circulating levels of pro-inflammatory cytokine IL-6, 24 hours after resuscitation in the Control group, which was significantly attenuated by PUGNAc, but not glucosamine (Sham: 8±6, Control: 181±36, PUGNAc: 42±22 pg/mL, $p<0.05$). Compared to the Sham group, the anti-inflammatory

cytokine IL-10 was significantly elevated only in the Control group, using Dunnett's posthoc-test ($p<0.05$) with an overall trend to significance (ANOVA, $p=0.08$). Compared to the Sham group, there were no significant differences in the serum levels of TNF-α in any group, 24 hours after TH-R.

Activation of Nuclear Factor kappa-B

In the liver, the binding affinity of NF-κB was significantly elevated in both Control and Glucosamine groups, 24 hours after TH-R; however, this was significantly attenuated in the PUGNAc group. There was no significant activation of NF-κB in either heart or lung.

Tissue iNOS levels

In the Control group, protein levels of iNOS were significantly increased compared to Sham surgery group in both liver and heart. This was significantly attenuated in the PUGNAc treated group, but not in the glucosamine treated group. Surprisingly, at this time-point, there was no significant induction of iNOS expression in the lung.

Myeloperoxidase (MPO) levels

Tissue MPO levels were significantly elevated in the Control animals in the lung 24 hours after TH-R, compared to Sham operated animals. However, neither Glucosamine nor PUGNAc treated groups showed significant elevation in MPO levels compared to Shams. In the heart and in the liver, there were no significant changes in the tissue MPO levels at this time-point.

Organ injury and apoptosis

PUGNAc significantly attenuated the trauma-hemorrhage-induced increase in serum levels of alanine transaminase (Sham: 95±14, Control: 297±56, PUGNAc: 126±21 IU, $p< 0.05$), aspartate transaminase (Sham: 536±110, Control: 1661±215, PUGNAc: 897±155 IU, $p < 0.05$), and lactate dehydrogenase (Sham: 160±18, Control: 1499±311, PUGNAc: 357±99 IU, $p<0.05$); however, glucosamine had no effect on these serum parameters. Indicators of pancreas (amylase) and kidney (creatinine / BUN) injury did not show significant changes at this time point in any group compared to Shams.

In the liver, there was an almost 2-fold increase of apoptosis in the Control group compared to the Sham group; however, neither Glucosamine, nor PUGNAc groups were significantly different from either the Sham or Control animals. In the lung, there was ~2.8 fold increase in apoptosis in the Control group compared to Sham group and this was attenuated in both Glucosamine and PUGNAc groups. In the heart, there was no significant change in apoptosis in any group compared to Shams.

Endoplasmic reticulum stress

GRP78 (glucose regulated protein) is a member of the heat shock protein family and an important indicator of endoplasmic reticulum stress. To evaluate the effects of TH-R and the

treatment protocols on ER stress, we assessed GRP78 levels in the liver, since of those organs studied, the liver appeared to be the most susceptible organ to injury and ER-stress. Here we found that there was no significant elevation of GRP78 (Bip) protein in the Control or any of treatment groups compared to Shams, 24 hours after TH-R.

Protein O-GlcNAc and phosphorlyation levels

In the liver, there was a significant, >50% decrease in overall O-GlcNAc levels 24 hours after TH-R, compared to the Sham group. PUGNAc treatment resulted in significantly elevated O-GlcNAc levels, compared to Control animals. However, glucosamine administration did not prevent the loss of O-GlcNAc (Fig. 4).

To determine whether there was a relationship between changes in global O-GlcNAc and phosphorylation levels, we examined both serine and threonine phosphorylation. Phosphorylation of serine residues was significantly elevated in both Glucosamine and PUGNAc treated groups compared to Controls; in PUGNAc group, serine phosphorylation was also significantly increased compared to the Sham group. In contrast, threonine phosphorylation of liver proteins was significantly decreased in PUGNAc group, compared to both Control and Glucosamine treated animals.

Furthermore, protein O-GlcNAc levels of the liver showed a significant negative correlation with indicators of tissue injury: ALT ($r=-0.71$, $p<0.05$), AST ($r=-0.75$, $p<0.05$), LDH ($r=-0.76$, $p<0.05$) and apoptosis ($r=-0.63$, $p<0.05$). There was also a modest, but significant correlation between O-GlcNAc levels at 24 hours and lactate levels ($r=-0.54$, $p<0.05$) measured at the end of resuscitation.

In the lung, we found a significant decrease in tissue protein O-GlcNAcylation levels in response to TH-R. Similarly to the liver, it was prevented by PUGNAc, but not glucosamine treatment. The protein O-GlcNAc changes followed a similar pattern in the heart, however, these changes were not significantly different (ANOVA, $p=0.07$).

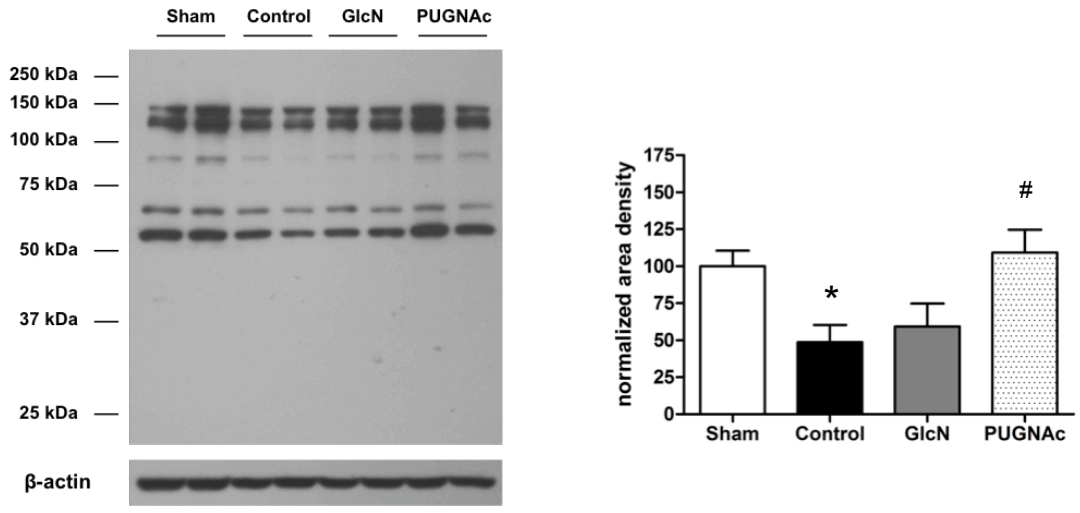

Fig. 4. O-GlcNAcylation in the Liver tissue extracts 24 hours after trauma-hemorrhage resuscitation *is shown on the left side. The representative blots show two-two selected samples of each group.* ***Bar chart*** *on the right side represents mean area densities of O-GlcNAc immunoblots, related to Sham animals. Mean densities were normalized to β-actin staining. Data mean ± SEM, n=7 in each group, ANOVA, * $p<0.05$ Vs Sham, # $p<0.05$ Vs Control.*

DISCUSSION

I. The effect of increased protein O-GlcNAc levels on the short-term outcome after severe trauma-hemorrhage in rats

Here we demonstrated for the first time that a bolus of glucosamine administered during hemorrhage significantly improved survival 2 hours following the end of hemorrhage (47% Vs 20%). Additionally, there was a significant increase in MAP in the glucosamine group for 18 minutes following treatment and consistent with our earlier study. We also found significantly higher protein O-GlcNAc levels in the heart, brain and liver extracts. Since both the mannitol and glucosamine solutions were of similar osmolarity, the improved survival in the glucosamine group is likely a direct effect of glucosamine.

Parallel with the progression of hypovolemic shock a metabolic acidosis develops as indicated by increased serum lactate accompanied by decreased pH, PO_2, BE and HCO3. While improvements in metabolic acidosis are typically associated with improved outcome following hypovolemic shock, thirty minutes following treatment there were no significant differences between the Control or Glucosamine treated groups in any of these parameters, suggesting that the improved survival in the glucosamine group cannot be attributed to an early reduction in metabolic acidosis or the severity of shock at that time.

Trauma combined with severe hemorrhage decreases cardiac output and peripheral tissue perfusion. This leads to activation of neuroendocrine reflexes resulting in centralized circulation with peripheral vasoconstriction. Here we found that glucosamine treatment resulted in a significantly increased MAP for a short duration immediately following treatment. In the absence of any other metabolic or hemodynamic effects, it is possible that this transitory increase in MAP was related to an acute vasoactive effect of glucosamine leading to further peripheral vasoconstriction. Alternatively, the increase in MAP could be due to an effect of glucosamine on the neuroendocrine response.

Maintenance of tissue bioenergetic status, particularly ATP levels could contribute to improved survival; therefore we determined ATP levels 30 min after treatment, which was a time just before animals in the mannitol treated group started to drop out. Glucosamine treatment had no effect on ATP levels in any organs examined suggesting that that the improved survival in this group was not related to the improved bioenergetics.

We observed only slightly increased cytokine levels 30 minutes following treatment, while glucosamine had no effect on these levels. One reason for this discrepancy may be that cytokine release is increased primarily following delayed resuscitation, while an immediate resuscitation or no resuscitation had significant effect on the serum cytokines levels.

Although the increase in O-GlcNAc levels in heart, liver and brain 30 minutes following treatment does not demonstrate a causal relationship between survival and increased O-GlcNAc levels, it is consistent with the notion that elevated O-GlcNAc levels are associated with improved survival. Surprisingly, there were no significant differences in the protein O-GlcNAc levels between glucosamine and mannitol treated groups in those animals that survived for 2 hours or those that died at similar time points during the original experiment. Since the turnover of O-GlcNAc on proteins is relatively rapid, it is possible that 2

hours following treatment O-GlcNAc levels in the glucosamine treated group started return to baseline levels

The mechanism(s) underlying the protection associated with increased O-GlcNAc levels remains to be determined. Meanwhile, it should also be noted that changes in O-GlcNAc have been shown to play an important role in modulating signal transduction pathways including modulation of p42/44 and p38 mitogen activated protein kinase (MAPK) activities and upstream MAPK kinases activity. Increased O-GlcNAc levels have also been shown to increase the expression of heat shock proteins (HSP) 70 and 40.

Small-or limited volume fluid resuscitation strategies are preferred for the treatment of hemorrhagic shock in combat operations or in the pre-hospital phase prior to surgical intervention, when large-volume resuscitation promotes further blood loss. The dose of glucosamine used here would translate to 150-250 mL / 70 kg b.w. or less if a more concentrated solution was used and this volume could be easily transportable by a first responder. Although oral administration yields serum glucosamine concentrations that are well below the millimolar range reported to have a cytoprotective effect, it does demonstrate an intrinsic lack of toxicity associated with glucosamine. While oral administration is different than intravenous treatment, 5 hours infusion of glucosamine at 4 μmol/dL/min resulting in local plasma glucosamine concentrations in the 0.4-0.8 mM range, were well tolerated with no adverse effects in 20 healthy young volunteers.

II. The effect of increased proteins O-GlcNAc levels on later outcome after severe trauma-hemorrhage in rats

Here we have demonstrated that enhancing O-GlcNAc levels by either increasing substrate availability with glucosamine or by inhibiting degradation with PUGNAc markedly improved 24 hour survival following severe trauma-hemorrhage and resuscitation in rats. While both treatments improved survival, only PUGNAc attenuated the hemorrhagic shock-induced pro-inflammatory responses and end organ injury.

In our treatment protocol, both glucosamine and PUGNAc were first administered immediately after hemorrhage with additional treatments during resuscitation. This approach was used in an attempt to model real-life conditions, where responding paramedics or battlefield life-savers could initiate treatment with a single i.v. dose. The pressure controlled hypovolemic phase models the subsequent transport, and the resuscitation phase represents the treatment provided in ICU or Primary Battalion Station / Forward Surgery Setting.

Of particular significance, we found that both glucosamine and PUGNAc administration improved survival rates (85% and 86%, respectively) compared to untreated Control animals (53%). Since the majority of deaths occurred over the first 6 hours, the increased survival and improved blood gas parameters in the treatment groups was most likely a consequence of an early protective effect. This is also consistent with the fact that serum lactate levels and blood gas parameters (BE, $HCO3^{-}$, TCO_2), indicators of shock severity, showed an early improvement in both glucosamine and PUGNAc treated groups. It is also worth noting that patients who subsequently develop MODS exhibit an early acidotic state at the time of admission, compared to patients who do not progress to MODS.

In contrast with the improved survival and early beneficial effects on blood gas parameters in both treatment groups, PUGNAc, but not glucosamine prevented the majority

of pro-inflammatory responses and attenuated the development of organ injury at the 24-hour time point.

Overall O-GlcNAc levels were markedly decreased in the lung and liver 24 hours after TH-R, while only PUGNAc administration resulted in maintained O-GlcNAc levels. Notably, O-GlcNAc modification occurs on Ser / Thre residues of proteins, similarly to phosphorylation While certain proteins are targeted exclusively by O-GlcNAcylation or phosphorylation, they are likely to exist in all possible combinations in a complex cellular environment. In our experiments, in the liver, O-GlcNAcylation levels changed in parallel with serine phosphorylation, but were reciprocal to threonine phosphorylation following TH-R.

While the loss of O-GlcNAc levels following resuscitation is consistent with our previous studies, it appears to contradict the notion that increased O-GlcNAc formation is a stress-induced response. However, studies have shown that O-GlcNAc levels increase relatively rapidly in response to ischemia and hypoxia, followed by a later decrease during reperfusion. In our current study, we also found the agonist induced increase in O-GlcNAc levels negatively correlates with indicators of tissue injury, apoptosis and serum lactate level after TH-R.

The mechanisms contributing to the stress induced changes in O-GlcNAc have yet to be determined; however, they are likely a result of changes in the activities of either OGT, O-GlcNAcase or both, combined with alterations in substrate availability mediated via the hexosamine biosynthesis pathway. The fact that increasing O-GlcNAc synthesis with glucosamine was less protective than inhibiting O-GlcNAc removal with PUGNAc, may reflect a shorter biological half-life for glucosamine. Alternatively, it may indicate that the loss of O-GlcNAc is indeed, due to an increase in O-GlcNAcase activity, which is blocked by PUGNAc. Nevertheless, these findings support the notion that the improved survival is a consequence of early events where O-GlcNAc levels are maintained by both glucosamine and PUGNAc treatment, while the decrease in the longer-term inflammatory response and tissue injury is result of the longer lasting effect of PUGNAc.

To demonstrate a direct cause-effect relationship between increased O-GlcNAc levels and survival the use transgenic animals could be valuable. However, OGT gene deletion is embryonically lethal, and OGT overexpression induces insulin resistance. Tissue specific conditional transgenic animals or acute adenoviral mediated enzyme delivery are potential alternative approaches; however, as hypovolemic shock affects all vital organs, it is unclear at this time which tissues or organs should be targeted. New, more selective O-GlcNAcase (NAG-thiazolines) and OGT (TTO4) inhibitors have been recently synthesized, and provide a more specific approach for future studies. These NAG-thiazolines have already been proven to increase significantly protein O-GlcNAc levels in isolated perfused hearts.

Importantly, the IgM-based monoclonal CTD 110.6 antibody recognizes the O-GlcNAc moiety itself, therofore; the immunoblots show several O-GlcNAcylated protein bands. Recently, new monoclonal O-GlcNAc specific IgG antibodies have been introduced, which can be used not only for detection, but immunoprecipitation and site localization. It is also important to note that, as is evident from the O-GlcNAc immunoblots, numerous proteins are targets for O-GlcNAc modification. The efficacy of the new IgG monoclonal antibodies has been demonstrated by large-scale enrichment of O-GlcNAc–modified proteins followed by shotgun proteomics, which led to the identification of more than 200 mammalian O-GlcNAc–modified proteins, including a large number of new glycoproteins. More studies are currently underway to identify proteins that exhibit changes in O-GlcNAc levels both in response to TH-R as well as following glucosamine and PUGNAc treatment.

NOVEL FINDINGS

» **The effect increased O-GlcNAc levels on the short-term outcome after severe trauma-hemorrhage in rats**

- Here we demonstrated that a bolus of glucosamine administered during hemorrhage significantly improved survival 2 hours following the end of hemorrhage in a minimal resuscitation model of severe trauma-hemorrhage.

- Additionally, there was a significant increase in mean arterial pressure in the glucosamine group for 18 minutes following treatment.

- We also found that glucosamine administration resulted in a significantly higher protein O-GlcNAc levels in the heart, brain and liver extracts.

» **The effect of increased proteins O-GlcNAc levels on later outcome after severe trauma-hemorrhage and resuscitation in rats**

- We have shown that enhancing O-GlcNAc levels by either increasing substrate availability with glucosamine or by inhibiting degradation with PUGNAc markedly improved 24 hour survival following severe trauma-hemorrhage and resuscitation in rats.

- Both treatments improved survival, however, only O-GlcNAcase inhibitor PUGNAc attenuated significantly the hemorrhagic shock-induced pro-inflammatory responses, apoptosis and end organ injury.

- The overall O-GlcNAc levels were markedly decreased in the lung and liver 24 hours after trauma-hemorrhage and resuscitation, while PUGNAc administration resulted in maintained O-GlcNAc levels.

- In the liver, the O-GlcNAcylation levels changed in parallel with serine phosphorylation, but were reciprocal to threonine phosphorylation 24 hours following trauma-hemorrhage and resuscitation.

- Additionally, we have found that increased O-GlcNAc levels in the liver showed a significant negative correlation with the serum lactate levels and the indicators organ injury.

In summary, our results demonstrate that metabolic interventions designed to increase protein *O*-GlcNAc levels may represent a valuable novel approach for the treatment hemorrhagic shock. Furthermore, increasing protein *O*-GlcNAc levels may also help to prevent the development of SIRS and the resulting MODS, decreasing the mortality of severe injuries.

PUBLICATIONS AND CONFERENCES

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Cumulative impact factor:	**35.793**
Citations:	**60**
Citations (without self-citation):	**41**

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Cumulative impact factor of listed abstracts: 51.550

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