Glucosamine improves cardiac function following trauma-hemorrhage by increased protein O-GlcNAcylation and attenuation of NF-κB signaling

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HYPONOLEMIA DUE TO HEMORRHAGE is a key factor in nearly half of the 150,000 deaths per year attributed to traumatic injury (34), and hemorrhage remains the primary cause of death on the battlefield in conventional warfare (2). Early deaths are determined by severe hemorrhage and central nervous system injuries, whereas late deaths are associated with inflammatory-mediated events, the development of sepsis, and multiple organ failure (1, 21, 37, 41). Multiple organ failure is typically characterized by the failure of at least two organ systems, such as lung, liver, and the cardiovascular system; the latter is associated with an especially high mortality rate (39). In a rat model of trauma-hemorrhage (T-H), we have demonstrated that treatment with glucosamine during resuscitation significantly improved cardiac function and peripheral organ perfusion and decreased the circulating levels of proinflammatory cytokines TNF-α and IL-6 (50).

Glucosamine is metabolized via the hexosamine biosynthesis pathway leading to the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is a substrate for multiple glycosylation reactions catalyzed by various GlcNAc transferases, including a unique O-linked GlcNAc (O-GlcNAc) transferase (OGT) (8, 13). In contrast to all other protein glycosyl transferases, OGT is found in the nucleocytoplasmic compartment rather than the endoplasmic reticulum where it catalyzes the formation of a reversible posttranslational protein modification involving the attachment of GlcNAc via an O-linkage to specific serine and threonine residues. In mammalian cells, a variety of stress stimuli have been shown to increase the level of O-GlcNAc on nuclear and cytoplasmic proteins (52). The inhibition of this response increased sensitivity to stress, whereas the augmentation of the O-GlcNAc levels increased the tolerance to the same stress stimuli and improved cell survival (52). We have previously reported that increasing O-GlcNAc levels in both isolated cardiomyocytes and the intact heart improved tolerance to ischemic injury (7, 14, 24, 25).

Following T-H, glucosamine treatment not only improved cardiac function but also increased O-GlcNAc levels in multiple tissues, including the heart (50). This raised the possibility that the effect of glucosamine could be mediated via its effect on O-GlcNAc synthesis; however, since glucosamine also increases glucosamine-6-phosphate and UDP-GlcNAc levels, its effect could be mediated via a number of other pathways. Supporting the notion that increasing O-GlcNAc levels contributed to the protection seen with glucosamine following T-H, we showed that O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc), an inhibitor of O-GlcNAcase, the enzyme that catalyzes the removal of O-GlcNAc from proteins, also increased tissue O-GlcNAc levels when administered during resuscitation and had a similar effect to glucosamine, improving cardiac function, as well as decreased the circulating levels of TNF-α and IL-6 (54).

A number of studies have shown that T-H-induced cardiac dysfunction was associated with increased TNF-α levels (32,
The nuclear factor-κB (NF-κB) signaling pathway plays a central role in regulating the release of many cytokines, including TNF-α and IL-6 (22, 31), and the activation of NF-κB has been implicated in organ dysfunction resulting from T-H (30, 32, 49). Therefore, the goal of this study was to test the hypothesis that the protection associated with glucosamine treatment during resuscitation was due at least, in part, to the attenuation of NF-κB signaling and that this was mediated via an increase in protein O-GlcNAcylation. We found that the in vivo administration of glucosamine following T-H attenuated the T-H-induced activation of NF-κB signaling in the heart and that glucosamine blocked LPS-induced TNF-α and IL-6 synthesis in isolated cardiomyocytes. We also demonstrate that OGT overexpression mimicked the effects of glucosamine treatment, whereas the transfection of cardiomyocytes with OGT small-interfering (si)RNA decreased O-GlcNAcylation levels and enhanced the response to LPS. Taken together, the results from these studies demonstrate that the modulation of O-GlcNAcylation alters the response of cardiomyocytes to the activation of the NF-κB pathway, which may contribute to the protection associated with glucosamine treatment.

MATERIALS AND METHODS

T-H shock model. All animal experiments were approved by the University of Alabama Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

T-H shock was induced in fasted male adult Sprague-Dawley rats (300 ± 25 g) as previously described in detail elsewhere (50, 54). Briefly, soft-tissue trauma was induced via a ventral midline laparotomy, followed by hemorrhage induced by removing blood via the left femoral artery to a mean arterial pressure (MAP) of 35–40 mmHg. Following T-H, there was a significant decrease in cardiac function, increases O-GlcNAc, and attenuates NF-κB activation. Cardiac function and systemic hemodynamic parameters 2 h after the end of resuscitation were summarized in Table 1. Consistent with our earlier reports (50), MAP and cardiac contractility were depressed following T-H and resuscitation, and both were significantly improved in the glucosamine-treated group. Hemoglobin levels and hematocrit were significantly depressed in both T-H groups, but there were no differences between the vehicle- and glucosamine-treated T-H groups, indicating that a similar level of blood loss was achieved in both groups. It is noteworthy, however, that while in vehicle-treated T-H group plasma lactate levels were markedly elevated, this response was significantly attenuated in the glucosamine-treated T-H group, such that plasma lactate levels were similar to those in the sham-operated groups.

Following T-H, there was a significant decrease in O-GlcNAc levels and an increase in IkB-α phosphorylation that was associated with an increase in both nuclear levels of NF-κB and NF-κB DNA binding activity (Fig. 1) compared with those in the sham-operated controls. However, glucosamine treatment during resuscitation maintained O-GlcNAc levels and attenuated the increase in IkB-α phosphorylation and the subsequent NF-κB nuclear translocation. Consistent with the attenuation of NF-κB activation, glucosamine treatment also attenuated the T-H-induced increase in cardiac mRNA of TNF-α and IL-6, as well as ICAM-1 protein expression (Fig. 2). One consequence of an increase in ICAM-1 is neutrophil infiltration that can then lead to further injury. MPO activity was measured as an index of neutrophil infiltration, and, as shown in Fig. 2D, MPO activity increased fivefold 2 h after resuscitation, and this was significantly attenuated in the glucosamine-treated group.

RESULTS

Statistical analysis. Data are expressed as means ± SE and compared by one-way ANOVA and Tukey’s test. Statistically significant differences between groups were defined as P < 0.05.
Glucosamine and OGT overexpression attenuates NF-κB activation in cardiomyocytes. The results from our in vivo studies demonstrated that glucosamine treatment during resuscitation improved cardiac function and attenuated the activation of NF-κB signaling pathway in the heart. However, the decrease in cardiac NF-κB activation could be secondary to the decrease in systemic inflammatory mediators rather than a direct effect of glucosamine on the heart. Therefore, we examined the effect of glucosamine treatment on LPS-induced TNF-α and IL-6 release in isolated adult cardiomyocytes. Consistent with previous reports, LPS increased both TNF-α and IL-6 release (20) and this was significantly attenuated by glucosamine treatment (Fig. 4A). LPS treatment alone had no effect on the overall cytoplasmic O-GlcNAc levels; however, glucosamine plus LPS significantly increased O-GlcNAc levels compared with untreated control cells (2.9 ± 0.3 vs. 1.7-fold increase in OGT expression compared with controls). Both glucosamine and OGT overexpression (Fig. 5) were also attenuated the LPS-induced increase in OGT-mediated increase in O-GlcNAc was associated with the attenuation of the LPS-induced increase in ICAM-1, and TNF-α expression and this was significantly attenuated by glucosamine treatment (Fig. 4A). LPS treatment alone had no effect on the overall cytoplasmic O-GlcNAc levels; however, glucosamine plus LPS significantly increased O-GlcNAc levels compared with untreated control cells (2.9 ± 0.7 vs. 1.0 ± 0.3, P < 0.05) (Fig. 4A).

NRVMs transfected with OGT adenovirus exhibited a 6.9 ± 1.7-fold increase in OGT expression compared with controls (P < 0.05), and this was associated with a 4.2 ± 0.8-fold increase in O-GlcNAc levels (P < 0.05) (Fig. 5A). Similar to glucosamine treatment, the OGT-mediated increase in O-GlcNAc was also associated with the attenuation of the LPS-induced increase in ICAM-1 phosphorylation, ICAM-1, and TNF-α expression (Fig. 5B). Both glucosamine and OGT overexpression also attenuated the LPS-induced increase in NF-κB (Figs. 4B and 5B), and this was associated with a two- to threefold increase in nuclear O-GlcNAc levels. One limitation of these experiments is that an empty adenoviral vector was not used as a control. However, since both glucosamine and OGT adenoviral transfection resulted in a similar blunted response to LPS-induced activation of NF-κB, we believe it is highly unlikely that the results from OGT overexpression are a consequence of nonspecific effects due to adenoviral transfection.

It should be noted that while we did not directly assess apoptosis, LPS treatment had no obvious impact on cell via-

### Table 1. Alterations in MAP, HR, ±dP/dt, and Hb at 2 h after T-H and resuscitation

<table>
<thead>
<tr>
<th></th>
<th>Sham-V</th>
<th>Sham-G</th>
<th>TH-V</th>
<th>TH-G</th>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>126.8±2.7</td>
<td>122.6±1.8</td>
<td>46.7±8.4*</td>
<td>81.5±4.4†</td>
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<tr>
<td>HR, beats/min</td>
<td>408.8±2.6</td>
<td>406.0±6.5</td>
<td>277.3±19.5*</td>
<td>386.1±15.0†</td>
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<tr>
<td>–dP/dt, mmHg/s</td>
<td>16,910±2,108</td>
<td>17,063±328</td>
<td>6,388±745*</td>
<td>11,619±712†</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>14.2±0.4</td>
<td>14.3±0.5</td>
<td>6.7±0.2*</td>
<td>5.8±0.4*</td>
</tr>
<tr>
<td>Hct, %</td>
<td>42.4±1.7</td>
<td>43.9±1.4</td>
<td>21.0±0.5*</td>
<td>18.4±1.2*</td>
</tr>
<tr>
<td>Lactate, mg/dl</td>
<td>10.2±0.5</td>
<td>6.8±0.7</td>
<td>73.4±17.3*</td>
<td>10.8±1.4†</td>
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Values are means ± SE (n = 5 rats/group). Sham-V, sham-operated rats (with vehicle); sham-G, sham-operated rats plus glucosamine; TH-V, trauma-hemorrhage (TH) treated with vehicle; TH-G, TH treated with glucosamine; MAP, mean arterial blood pressure; HR, heart rate; –dP/dt, maximum change of pressure over time; −dP/dt, minimum change of pressure over time; Hb, hemoglobin; Hct, hematocrit. Values were compared by one-way ANOVA and Tukey's test: *P < 0.05 vs. sham-V; †P < 0.05 vs. TH-V.
bility over the time course of these experiments in either adult or neonatal cardiomyocytes (data not shown).

Decreased OGT expression enhances the response to LPS. Transfection of NRVMs with OGT siRNA significantly decreases O-GlcNAc levels by 60% and OGT levels by 50% compared with scrambled siRNA cells, and this response was unaffected by LPS treatment (Fig. 6, A and B). Consistent with the results in Figs. 4 and 5, LPS treatment significantly increased IκB-α phosphorylation; however, this response was markedly augmented in the OGT siRNA group (Fig. 6, A and C). Glucosamine attenuates response of macrophages to LPS. Macrophage activation is an important contributing factor not only to the systemic inflammatory response seen following T-H and resuscitation but also to the subsequent progression of tissue injury. Therefore, we examined the effect of glucosamine treatment on the LPS-induced IκB-α phosphorylation and inducible nitric oxide synthase (iNOS) expression in the macrophage cell line, RAW 264.7. As shown in Fig. 7, similar to the results in cardiomyocytes, LPS-induced IκB-α phosphorylation was attenuated by glucosamine treatment, and this was associated with increased O-GlcNAc levels. As expected, LPS markedly increased iNOS expression, and this was also substantially attenuated with glucosamine treatment.

DISCUSSION

Advances in intensive care and patient management have improved short-term survival following severe injury (4, 40); however, the overall hospital mortality for adult trauma patients admitted to the intensive care unit is almost 20% (46). Cardiac dysfunction is a frequent complication associated with severe trauma and is an important factor contributing to the high mortality associated with multiorgan failure (39). Rodent models of hemorrhagic shock recapitulate many of the responses seen in patients, including depressed cardiac function and impaired peripheral organ perfusion and function in addition to increased inflammatory response (10, 49). We have previously reported that following T-H increasing tissue levels of O-GlcNAc, either by augmenting synthesis with glucosamine or inhibiting degradation with the O-GlcNAcase inhibitor PUGNAc, improved cardiac function and peripheral organ perfusion, decreased end-organ injury, and reduced the circulating levels of inflammatory cytokines (50, 54). However, the mechanism underlying this protection had not been identified. We show here, for the first time, that an in vivo administration of glucosamine during resuscitation markedly attenuated the activation of the NF-κB pathway in the heart and that this can be replicated by glucosamine treatment of isolated cardiomyocytes.

The fact that glucosamine and PUGNAc, both of which increase O-GlcNAc levels but via different mechanisms, improved the outcomes following T-H and resuscitation in vivo (50, 54), support the notion that the observed protection is
mediated via O-GlcNAc. Nevertheless, glucosamine treatment can affect a number of pathways other than O-GlcNAc (29, 45), and PUGNAc can also inhibit other β-hexosaminidases (28, 38) and thus may alter the processing of glycoconjugates in addition to O-GlcNAc. Therefore, we also examined whether altering cardiomyocyte OGT expression would modulate NF-κB activation and found that increasing OGT levels mimicked the effects of glucosamine, not only increasing O-GlcNAc levels but also attenuating LPS-induced activation of NF-κB. Conversely, decreasing OGT expression significantly enhanced the response to LPS. Taken together, these results suggest that the improvement in cardiac function seen with glucosamine treatment following T-H is due at least in part to the attenuation of NF-κB signaling that follows severe trauma such as hemorrhagic shock. The activation of NF-κB plays an important role in the systemic increase in inflammatory mediators, including TNF-α and IL-6, which we have shown are both decreased by glucosamine and PUGNAc treatment. An excessive production of nitric oxide as a result of increased iNOS expression is another important factor contributing to both increased inflammation as well as end-organ injury that occurs following hemorrhagic shock (15). Thus, while additional studies are needed to demonstrate a more definitive link between O-GlcNAc and the regulation of NF-κB signaling in macrophages, these results suggest that increases in O-GlcNAc levels not only afford direct protection to parenchymal tissue but also attenuate the activation of systemic inflammatory responses to trauma.

In addition to attenuating NF-κB activation in cardiomyocytes, we also show preliminary evidence that glucosamine attenuates LPS-induced NF-κB activation in macrophages and that this is also associated with an increase in O-GlcNAc levels (Fig. 7). This observation suggests that the activation of O-GlcNAc formation will also attenuate NF-κB signaling in cells that contribute to the systemic inflammatory response that follows severe trauma such as hemorrhagic shock.
Much of the work examining the role of O-GlcNAc in regulating cellular function has been in the context of various chronic disease states (9, 11, 13, 27, 43, 47); however, it is increasingly clear that protein O-GlcNAcylation is an important mechanism involved in signal transduction and influences a wide range of cellular processes, including cell cycle, cell growth, apoptosis, and cell survival (27, 44, 51). The importance of O-GlcNAc synthesis in regulating cell survival was first reported by Zachara et al. (52); we have subsequently shown that ischemia-reperfusion increased O-GlcNAc levels in a glucose-dependent manner (7) and that the augmentation of this significantly improved cell survival, whereas the attenua-
O-GlcNAcylation and NF-κB signaling in the heart

Fig. 7. Glucosamine treatment of RAW 264.7 cells increased cardiac O-GlcNAc levels and attenuated LPS-induced P-IκB-α and inducible nitric oxide synthase (iNOS) protein expression. Cells were pretreated with 5 mM glucosamine for 30 min and before treatment with LPS (0.1 μg/ml, 6 h). C, control; L, LPS treated.

NF-κB is known to be activated by more than 400 different stimuli; here we used LPS, which initiates NF-κB activation via binding to Toll-like receptor-4 (26). While the upstream signaling pathways differ depending on the specific stimuli, in classical NF-κB signaling there is a convergence at the IkB kinase (IKK) complex leading to the phosphorylation of IkB-α. As we only examined one activator of NF-κB, it is possible that the O-GlcNAc-mediated attenuation of IkB-α phosphorylation is specific for LPS; however, a recent study in vascular smooth muscle cells demonstrated that increased O-GlcNAc levels attenuated the TNF-α-induced activation of NF-κB (48). This suggests that the effect of increased O-GlcNAcylation on NF-κB signaling is likely mediated at the level of, or downstream from, IKK.

While these data support the notion that O-GlcNAc regulates NF-κB activation, the question remains as to the mechanism by which this occurs. James et al. (18) reported that an increase in hexosamine biosynthesis pathway flux was associated with increased O-GlcNAc modification of the p65 subunit of NF-κB, which could be a potential mechanism. The nuclear translocation of NF-κB is dependent on the phosphorylation of IkB-α, which is subsequently targeted for degradation by the proteasome. Thus it is also possible that O-GlcNAcylation of IkB-α may prevent its phosphorylation, thereby preventing its degradation. Alternatively, increased O-GlcNAc levels decrease proteasome activity (53), which could decrease IkB-α degradation and thus indirectly attenuate NF-κB translocation. It should also be noted that the phosphorylation of serine and threonine residues of IKK is required for their activation, and the acetylation of these residues prevents phosphorylation and inhibits NF-κB signaling (33). Thus it is also possible that O-GlcNAcylation of IKK could also block the activation of NF-κB. Clearly, further studies are warranted to determine the mechanism(s) by which increased flux through OGT inhibits NF-κB activation.

In this study, we investigated only a single time point following T-H and resuscitation. It could be argued that investigating a single time point is not sufficient to conclude a potential role of glucosamine in the observed effects on NF-κB following T-H. However, previous studies have shown that if a pharmacological agent was effective in improving cell and organ function early after the insult, i.e., at 2 h after resuscitation, that agent also improved cell and organ function at later time points, i.e., 24 and 48 h, and that it also improved the survival of animals subjected to T-H and the induction of subsequent sepsis (3, 12, 42). Thus, based on these studies, it would appear that the salutary effects of glucosamine on NF-κB and other parameters would persist even if one examined those effects at 24 or 48 h after glucosamine treatment. In support of this, a recent study demonstrated that glucosamine administered during resuscitation improved 24-h survival rates following T-H (35). Interestingly, 24 h following resuscitation, in untreated animals, protein O-GlcNAc levels remained depressed in the liver and lung but not the heart, suggesting tissue-specific differences in O-GlcNAc turnover rates. Future studies looking at O-GlcNAc levels and NF-κB activation, in different tissues at different time points following resuscitation, would...
provide valuable insight into the role of O-GlcNacylation and the regulation of NF-κB signaling.

In conclusion, we have shown that the in vivo administration of glucosamine attenuates the activation of NF-κB in the heart induced by T-H and resuscitation, and this was associated with improved cardiac function, decreased systemic inflammatory response, and increased tissue O-GlcNAc levels. This was recapitulated at the cellular level where glucosamine attenuated LPS-induced activation of NF-κB and its downstream targets. We also demonstrated that an increased expression of OGT mimicked the effect of glucosamine both with regard to the attenuation of NF-κB as well as the increase in O-GlcNAc levels, supporting the notion that the response to glucosamine is mediated via increased flux through OGT and increased O-GlcNAc formation. A role for O-GlcNAc in mediating NF-κB phosphorylation was also found that glucosamine attenuated the LPS-induced phosphorylation of IkB-α phosphorylation and iNOS expression in macrophages, demonstrating that the in vivo administration of glucosamine may exert its protective effect not only at the level of the heart and other parenchymal tissues but also by the attenuation of inflammatory responses in circulating mediators such as macrophages. We have yet to identify the specific molecular mechanism by which changes in O-GlcNAc status modulate NF-κB activation; however, given the critical role of NF-κB in mediating the cardiovascular response to different stress stimuli, these results suggest that strategies designed to acutely increase O-GlcNAc levels may represent a valuable new therapeutic approach for not only T-H but also sepsis and ischemic injury.

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