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The Mechanisms and Physiological Relevance of Glycocalyx Degradation in Hepatic Ischemia/Reperfusion Injury

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Abstract

Significance: Hepatic ischemia/reperfusion (I/R) injury is an inevitable side effect of major liver surgery that can culminate in liver failure. The bulk of I/R-induced liver injury results from an overproduction of reactive oxygen and nitrogen species (ROS/RNS), which inflict both parenchymal and microcirculatory damage. A structure that is particularly prone to oxidative attack and modification is the glycocalyx (GCX), a meshwork of proteoglycans and glycosaminoglycans (GAGs) that covers the luminal endothelial surface and safeguards microvascular homeostasis. ROS/RNS-mediated degradation of the GCX may exacerbate I/R injury by, for example, inducing vasoconstriction, facilitating leukocyte adherence, and directly activating innate immune cells. **Recent Advances:** Preliminary experiments revealed that hepatic sinusoids contain a functional GCX that is damaged during murine hepatic I/R and major liver surgery in patients. There are three ROS that mediate GCX degradation: hydroxyl radicals, carbonate radical anions, and hypochlorous acid (HOCl). HOCl converts GAGs in the GCX to GAG chloramides that become site-specific targets for oxidizing and reducing species and are more efficiently fragmented than the parent molecules. In addition to ROS/RNS, the GAG-degrading enzyme heparanase acts at the endothelial surface to shed the GCX. **Critical Issues:** The GCX seems to be degraded during major liver surgery, but the underlying cause remains ill-defined. **Future Directions:** The relative contribution of the different ROS and RNS intermediates to GCX degradation *in vivo*, the immunogenic potential of the shed GCX fragments, and the role of heparanase in liver I/R injury all warrant further investigation. *Antioxid. Redox Signal.* 21, 1098–1118.

Introduction

MICROVASCULAR DYSFUNCTION LIES at the basis of various prevalent and life-threatening conditions, such as diabetes, hypertension, and ischemia/reperfusion (I/R) injury (40, 89, 162). In all cases, the endothelial cells (ECs) that line the microvasculature are attacked and damaged by reactive oxygen and nitrogen species (ROS and RNS, respectively) (13, 50, 153) that are overproduced by ECs and several types of chemoattracted immune cells. Moreover, the ROS/RNS-mediated modification of extracellular and intracellular EC constituents converts the endothelium to a proinflammatory surface through which the immune response is propagated,

culminating in a self-amplified cycle of microvascular dysfunction and tissue injury.

One of the most oxidation-sensitive components of ECs is the glycocalyx (GCX) (153), which comprises a network of proteoglycans (PGs), glycosaminoglycans (GAGs), and glycoproteins that covers the luminal endothelial surface (164). The GCX forms a functional barrier between blood and the endothelium that maintains microvascular homeostasis by regulating vascular permeability (150), vascular tone (147), and leukocyte adherence (129). As was initially demonstrated in animal models of hyperglycemia and type II diabetes (177), the GCX can be used as a prognostic tool to gauge the clinical course of patients with a vascular

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disorder (18, 100), underscoring the importance of the GCX.

In the surgical setting, the manifestation of microvascular dysfunction, inflammation, and oxidative/nitrosative stress is prominent in liver surgery-induced I/R, which occurs during liver transplantation and liver resection (154, 173). The recovery of the liver during early reperfusion is known to be impaired by microvascular defects caused by vasoconstriction and leukocyte plugging (162), which hamper the oxidative phosphorylation-dependent repletion of energy levels (*i.e.*, adenosine triphosphate [ATP] and guanosine triphosphate [GTP]) that are needed to support the elevated metabolic demand in post-ischemic hepatocytes (72). This culminates in an initial wave of hepatocellular demise and the subsequent release of immunogenic particles known as damage-associated molecular patterns (DAMPs), the onset of a sterile immune response (154), and an increase in hepatic oxidative/nitrosative stress (153). During the later reperfusion phase, the microvasculature acts as a biological scaffold for the docking and transmigration of chemoattracted leukocytes that further debilitate liver function as a result of ROS/RNS production and consequent parenchymal damage (154).

Many of the phenomena that contribute to the hepatopathology following I/R can be traced back to the loss of GCX integrity and function. Despite the unequivocal clinical relevance of GCX degradation (98, 101, 102, 160), the mechanistic foundation of GCX shedding in most pathological settings remains poorly understood. In this review, the following subjects will therefore be addressed in the context of hepatic I/R injury: the structure–function relationship of the GCX, the mechanisms of GCX degradation by ROS/RNS, and the inflammatory consequences of oxidative/nitrosative modification of the GCX in relation to liver injury.

GCX Structure–Function Relationship

GCX structure

The core of the GCX is composed of syndecan and glypican PGs, which either have an EC membrane-spanning domain (syndecan) or are connected to the endothelium *via* a glycosyl phosphatidylinositol anchor (glypican) (124, 164). The PGs form a solid backbone to which GAG side chains are covalently attached. GAGs are long polysaccharides composed of fixed pairs of alternating monosaccharides. Whereas glypicans are exclusively substituted with heparan sulfate (HS), syndecans carry both HS and chondroitin sulfate (CS) side chains (Fig. 1), as a result of which the HS to CS ratio in the GCX is $\sim 4:1$ (116). Notwithstanding these variations, GAGs always attach to a similar amino acid sequence in the PG core, which is a serine–glycine repeat located in a region rich in acidic amino acids (174, 175).

The branching pattern of GAGs is tightly regulated by glycosyltransferases and sulfotransferases (17, 36). First, a tetrasaccharide (xylose–galactose–galactose–glucuronic acid) is conjugated to the serine in the PG (17, 36), which is followed by the addition of either *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) to the glucuronic acid (GluA) of the tetrasaccharide, giving rise to HS or CS, respectively (Fig. 2). Once the chain has been elongated, HS and CS undergo post-translational modifications, including the sequential deacetylation and sulfation of the GlcNAc amino group (HS) and variable O-sulfation of the GlcNAc and GluA ring (HS and CS, Fig. 2) (17, 36). To add to the complexity, the GluA of HS and CS can be converted to iduronic acid (IduA) by glucuronyl C-5 epimerase (79).

Another major GAG of the GCX is hyaluronic acid (HA), which has a more uniform structure and is built up of unsubstituted GlcNAc–GluA repeats (Fig. 2). In contrast to HS and CS, HA is not linked to a PG core but rather inserts into

FIG. 1. GCX structure. The backbone of the GCX is composed of syndecan and glypican PGs that are anchored in the endothelial cell membrane. Long sulfated GAG (HS [in green] and CS [in orange]) side chains are covalently attached to the PG cores. The nonsulfated GAG HA (in red) is not bound to a PG but attaches to the GCX by directly associating with CS chains or endothelial surface receptors such as CD44 (not shown). CS, chondroitin sulfate; GAG, glycosaminoglycan; GCX, glycocalyx; HA, hyaluronic acid; HS, heparan sulfate; PG, proteoglycan. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 2. GAG structure. GAGs are attached to the proteoglycan core via a tetrasaccharide linkage composed of Xyl, Gal (2×), and GluA. Next, HS or CS are produced by respectively adding GlcNAc or GalNAc to the GluA of the tetrasaccharide linker. X and Y indicate variable substitution of amino- or O-groups, respectively (see in-figure legend). Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GluA, glucuronic acid; Xyl, xylose. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the GCX by associating directly with CS chains *via* electrostatic interactions (149) or with the EC surface receptor CD44 (83).

GCX size

The reported functional width of the GCX varies per site and type of blood vessel and is best measured using intravital fluorescence imaging techniques (137, 158), which take the hydrodynamic properties of the GCX into account. *In vivo*, the GCX size ranges from $\sim 0.5 \mu\text{m}$ in mouse and hamster cremaster muscle capillaries (112, 159) to $\sim 0.75 \mu\text{m}$ in rat mesenteric venules (82) and $\sim 1.7 \mu\text{m}$ in mouse pulmonary microvessels (129). The size range increases ($< 0.1\text{--}4.5 \mu\text{m}$) when *ex vivo* imaging techniques such as immunofluorescence (85, 147) or electron microscopy (80, 150) are used. These methods, however, are more prone to artifacts in that either a portion of the GCX is lost during sample processing or the GCX unfolds from its compact *in vivo* conformation, which could result in an under- or overestimation of the functional GCX thickness, respectively [discussed in Ref. (127)]. It must be noted that the prerequisites to build up or maintain a functional GCX *in vivo* are not fully understood. In that respect, only a minute reduction in GCX thickness is observed in the cremaster muscle vasculature of syndecan-1 knockout mice (127), despite the fact that syndecan-1 is considered the most predominant EC surface PG in wild-type mice (124).

GCX function

The protective functions of the GCX emanate from its physical as well as chemical characteristics. Regarding the former, the width of the GCX greatly exceeds that of intercellular adhesion molecule 1 (ICAM-1), an important sinusoidal receptor for leukocytes (93) and platelets (66). ICAM-1 only spans 18.7 nm from the endothelial surface

(139), which explains why leukocytes and platelets generally do not immobilize on nonperturbed or noninflamed endothelium. Accordingly, the adherence of platelets (157) and leukocytes (88, 129) is greatly enhanced in pathological settings in which the GCX is compromised. The cytosolic domain of the membrane-spanning syndecans also contributes to GCX functionality through their connectivity with the EC actin cytoskeleton (147). ECs use this connection to translate information related to plasma drag on their luminal tips, which is relayed by syndecan, to the production of nitric oxide ($\bullet\text{NO}$) by endothelial nitric oxide synthase (eNOS) (38), thereby proportioning vascular tone to perfusion demands.

As for the chemical attributes of the GCX, the high degree of HS/CS sulfation and deprotonated GAG carboxylic acids at physiological pH (163) impart a net negative charge on the GCX, which, in combination with the high molecular density of the GCX, allows the GCX to adsorb or exclude plasma macromolecules based on both charge and size. This molecular sieve-like property of the GCX controls plasma colloid oncotic pressure and thereby fluid exchange between the (micro)circulation and the interstitial space. The barrier function of the GCX, however, is easily perturbed. Perfusing rat renal arteries with a hypertonic sodium chloride solution to displace only noncovalently bound proteins from the GCX induced a rapid rise in endothelial permeability, which ultimately led to proteinuria (42). The observation that the GCX maintains the endothelial filtration barrier has even prompted a revision of the Starling principle (78), which now includes that perturbation of the GCX drastically increases net fluid filtration.

The GCX additionally harbors several types of proteins to safeguard microvascular homeostasis and integrity, as is exemplified by the antioxidant enzyme extracellular superoxide dismutase (ecSOD). ecSOD binds electrostatically to sulfated GAGs (60, 75) and to a certain extent protects the GCX from

free radical-mediated degradation by converting superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). Although the nonradical H_2O_2 is a less potent oxidant than $O_2^{\bullet-}$, it can catalyze decomposition of the GCX *via* the formation of free radical derivatives (see the Mechanisms of GCX Oxidation section). Loss of GCX-related physiological functions, which results in the adherence of leukocytes and platelets (72, 134), vasoconstriction and microcirculatory defects (162), oxidative stress (72), and interstitial edema (71), are all prominent features of hepatic I/R injury. These common denominators underscore the potential link between GCX degradation and hepatic I/R injury, as is schematically depicted in Figure 3.

Hepatic GCX: *in vivo* proof of concept

It has been previously shown that HA (83), CS (90), and syndecan-1 (138) are expressed in the hepatic sinusoids (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars), although it remains to be experimentally confirmed that these components in fact form a functional GCX. To investigate the presence of a functional GCX in the liver, intravital two-photon microscopy was used to visualize the hepatic GCX in mice using the fluorescent dye exclusion technique validated by Vink and Duling (158,159). By infusing two types of fluorescently labeled dextrans that differ in size, surface charge, and fluorescence emission spectra, causing one tracer to be excluded from the GCX (*e.g.*, green fluorescence) while the other penetrates into the GCX (*e.g.*, red fluorescence), the difference in diameter between the two fluorescent columns can be used to calculate the size of the GCX (Fig. 4). Preliminary experiments revealed that the median functional width of GCX in hepatic sinusoids is $0.193\ \mu\text{m}$ with an interquartile range of $0.329\ \mu\text{m}$ (Fig. 4A–D). The GCX seemed absent in larger vessels (Fig. 4E–G), which is in agreement with the reported lack of HA expression in post-sinusoidal mouse venules (83).

The GCX size determined in this study is lower than that reported for the GCX of pulmonary or cremaster muscle capillaries. Moreover, the GCX size seems to strongly fluctuate between the different hepatic vessels (Fig. 4D). These variations may be related to the fact that GAGs are heterogeneously distributed throughout the sinusoids (Supplementary Fig. S1), which probably is inherent to the fenestrated and discontinuous nature of the sinusoidal endothelium (136).

ROS and RNS in Hepatic I/R Injury

Hepatic I/R injury can be categorized into three distinct phases according to the site of most abundant ROS/RNS production during the reperfusion period (153). In the hyperacute phase (~ 0 –30 min reperfusion), hepatocytes resume oxidative phosphorylation in an attempt to restore energy levels (72), thereby fueling a burst of mitochondrial ROS production (87, 91). Cells that are unable to cope with this excessive mitochondrial ROS formation and that fail to replenish their ATP reserves predominantly go into necrosis (57), resulting in leakage of cellular content into the circulation. The leaked content contains DAMPs that alert the immune system of (impending) tissue damage (151). Subsequently, DAMPs initiate the second reperfusion phase (~ 30 min–6 h, acute phase) by stimulating liver-resident macrophages (Kupffer cells [KCs]) to produce ROS/RNS and release proinflammatory cytokines and chemokines (151). As a result, a sterile immune response is triggered that marks the third reperfusion phase (~ 6 –24 h, chronic phase), during which chemoattracted leukocytes account for the bulk of ROS/RNS production (153), putting the GCX at an increased risk for oxidative/nitrosative degradation. For a more detailed overview of the role of ROS/RNS in the pathophysiology of hepatic I/R injury, the reader is referred to recent reviews (56, 153, 173).

FIG. 3. Schematic representation of GCX degradation during hepatic I/R injury. An intact GCX (shown on the left) maintains vascular homeostasis by, for example, preventing leukocyte adherence (see the GCX Function section). During reperfusion, the production of ROS/RNS (green) by SECs and leukocytes (*e.g.*, monocytes, neutrophils) and the release of heparanase by SECs (yellow) could lead to GCX degradation. The loss of GCX not only abrogates the protective functions of the GCX but additionally activates the immune system (shown on the right). Circulating GCX fragments can be detected by immune receptors on the surface of Kupffer cells and SECs, thereby inciting the production of proinflammatory mediators such as $TNF-\alpha$ (see the Proinflammatory Consequences of GCX Degradation section). I/R, ischemia/reperfusion; RNS, reactive nitrogen species; ROS, reactive oxygen species; SEC, sinusoidal endothelial cell; $TNF-\alpha$, tumor necrosis factor alpha. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 4. Intravital two-photon microscopy of the hepatic GCX in mice. (A–C) Show the hepatic sinusoids as visualized by intravital two-photon microscopy in male C57Bl/6 mice ($N=2$) following the intravenous infusion of a neutral 40-kDa Texas Red-dextran [(A), red fluorescence] and an anionic 150-kDa FITC-dextran [(B), green fluorescence]. The overlay of (A) and (B) is shown in (C). The fluorescent column diameters are shown in the insets that correspond to the region delineated by the dashed marquee. Inasmuch as the smaller neutral (red) dye penetrates into the GCX, whereas the larger and anionic (green) dye is excluded by the GCX (159), the width of the GCX per vessel can be measured by subtracting the diameter of the FITC (green) fluorescence column from the diameter of the Texas Red (red) fluorescence column and dividing this number by 2. The *inset* in (A) shows a representative measurement of a Texas Red fluorescence column, which is superimposed on the FITC fluorescence column at the exact same position in (B) to give the composite image in (C), revealing a diameter difference that is most likely attributable to FITC-dextran exclusion by the GCX. This is further illustrated in the overlay (C), where it appears that the hepatic sinusoids are lined by exclusively red fluorescence (*arrowheads*), again indicating that the green dye does not penetrate to the vascular wall due to spatial exclusion by the GCX. (D) Shows the calculated GCX width for 44 regions of interest, yielding a median GCX size of $0.193\ \mu\text{m}$ (interquartile range = $0.329\ \mu\text{m}$). Error bars (shown in red) also represent the median and interquartile range. The results were validated by an observer that was blinded to the experimental design (Supplementary Fig. S2). (E–G) Indicate that the width difference between the two fluorescence columns seems absent in post-sinusoidal venules, implying that the GCX may only be present in the sinusoids. The intravital imaging procedure is described in detail in the Supplementary Data. FITC, fluorescein isothiocyanate. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Oxidative and Nitrosative Modification of the GCX

Although the evidence is limited, a causal relationship between ROS/RNS production and GCX degradation has been reported. In mouse cremaster muscle capillaries subjected to I/R, the acute (<5 min) loss of GCX upon reperfusion could be prevented by neutralizing $O_2^{\cdot-}$ and H_2O_2 with a combination of SOD and catalase (125). Inasmuch as pretreatment with allopurinol yielded similar results, the $O_2^{\cdot-}$ -generating enzyme xanthine oxidoreductase (XOR) was identified as the mediator of oxidative GCX damage, which is plausible considering that XOR tethers to sulfated GAGs on the endothelial surface (2, 113). Additionally, it was demonstrated that the addition of H_2O_2 to the culture medium results in the rapid (1–2 h) removal of HS (but not HA) from the surface of cultured human glomerular ECs, thereby compromising the endothelial barrier function (135). Notwithstanding the controversies surrounding *in vitro* GCX research (22, 111), the loss of cell surface HS occurred without affecting cell viability (135), suggesting that the GCX is already at risk for oxidative degradation by relatively benign oxidants and at relatively low ROS concentrations.

Although these results provide seminal proof-of-principle regarding ROS as catalysts of GCX fragmentation, the mechanistic foundation of these effects is incompletely understood. Nevertheless, several scenarios can be outlined that are detrimental to the GCX when taking into account the temporal organization of hepatic I/R injury, the known sources of ROS/RNS, and the chemical properties of the reactive species that are generated.

Mechanisms of GCX oxidation

Based on the reaction rates of the various ROS/RNS with GCX-pertinent GAGs (4–6, 63–65, 109, 117, 118, 132, 133), there are three biologically relevant radical species that directly induce GAG chain scission, albeit *via* distinct routes: hydroxyl radicals ($\cdot OH$), carbonate radical anions ($CO_3^{\cdot-}$), and hypochlorous acid (HOCl). All three species comprise secondary and/or tertiary derivatives of the template oxidants $O_2^{\cdot-}$ and $\cdot NO$ (Fig. 5). The production of $\cdot OH$ and $CO_3^{\cdot-}$ is preceded by the formation of the peroxynitrite anion ($ONOO^-$), which is generated in the diffusion-controlled reaction between $O_2^{\cdot-}$ and $\cdot NO$ and is in balance with its conjugate acid peroxynitrous acid ($ONOOH$) ($pK_a=6.8$) at physiological pH (Fig. 5). Whereas $CO_3^{\cdot-}$ (together with nitrogen dioxide [$\cdot NO_2$]) is formed in a reaction between $ONOO^-$ and carbon dioxide (CO_2) (31, 46), $\cdot OH$ is generated through homolytic fission of $ONOOH$ (14). Given that the $ONOO^-/ONOOH$ balance shifts toward the former at physiological pH and that CO_2 is freely available, the decomposition of $ONOO^-/ONOOH$ should favor the formation of $CO_3^{\cdot-}$ over $\cdot OH$ during hepatic I/R injury. Alternatively, $\cdot OH$ can be produced in the reaction between $O_2^{\cdot-}$ and myeloperoxidase (MPO)-derived HOCl (see the Nonresident Leukocytes section) (114) or through a Fenton reaction involving H_2O_2 and ferrous iron (Fe^{2+}) (37) (Fig. 5). However, the latter reaction is most likely a less significant route to $\cdot OH$ production in biological systems inasmuch as iron normally circulates as ferric iron (Fe^{3+}) in a transferrin-bound state that prevents it from participating in metal-catalyzed reactions (45). Nevertheless, the release of redox-active transition metals (TMs, *i.e.*, Fe^{2+} and Cu^+) from circulating protein-bound

FIG. 5. The formation of ROS and RNS during hepatic I/R injury. The combined presence of the template radicals $\cdot NO$ and $O_2^{\cdot-}$ ultimately gives rise to three species, shown in red, that directly induce GCX modification or fragmentation: $CO_3^{\cdot-}$, $\cdot OH$, and HOCl. The enzymatic and metal catalysts are indicated in green, see the Mechanisms of GCX Oxidation section for details. $\cdot OH$, hydroxyl radical; $\cdot NO$, nitric oxide; $CO_3^{\cdot-}$, carbonate radical anion; eNOS, (uncoupled) endothelial nitric oxide synthase; HOCl, hypochlorous acid; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NOX2, phagocyte NADPH oxidase; $O_2^{\cdot-}$, superoxide anion; SOD, superoxide dismutase; TM, transition metal. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

stores such as ferritin (16), hemoglobin (81), and ceruloplasmin (140) has been observed in settings of severe oxidative/nitrosative stress. Because free TM ions electrostatically bind to the GCX (11), they could mediate GCX decomposition by locally reacting with H_2O_2 to form $\cdot OH$. In contrast to aforementioned oxidants, HOCl is produced enzymatically in the reaction between MPO and H_2O_2 (24, 70, 103). Since neutrophils are the main source of MPO, HOCl is mainly produced in the chronic phase of I/R injury (see the ROS and RNS in Hepatic I/R Injury section).

Oxidative fragmentation of GAGs by biologically relevant radicals and nonradical halogen-lacking oxidants. Oxidative damage to GAGs has been linked to almost all biologically relevant radicals ($\cdot OH$, $CO_3^{\cdot-}$, $O_2^{\cdot-}$) and nonradical oxidants (HOCl, $ONOO^-/ONOOH$) (108, 120). For instance, ionizing radiation in combination with gel permeation chromatography (GPC)/multiangle laser light scattering (MALLS) has been used to measure the changes in molecular mass of polydispersed HA following exposure to radicals. With these techniques, HA fragmentation efficiencies of 52% and 20% were calculated for $\cdot OH$ and $CO_3^{\cdot-}$, respectively (6). In a later study, lower fragmentation efficiencies of 32% and 11% were found for $\cdot OH$ and $CO_3^{\cdot-}$ (Table 1), respectively, which could be attributable to the much lower molecular mass HA that was used (132). Kinetic analysis in pulse radiolysis experiments revealed that $CO_3^{\cdot-}$ reacted two or three orders of magnitude more slowly than the almost diffusion-controlled rates found for $\cdot OH$ (6, 133). This large difference suggests that $\cdot OH$ is random in its attack of HA, which has 11 potential hydrogen abstraction (H-abstraction) sites, and that $CO_3^{\cdot-}$ is more site-selective in its attacks (6).

The fragmentation of HA and other GAGs has also been investigated by Kennett and Davies by electron paramagnetic resonance (EPR) spectroscopy and sensitive polyacrylamide

TABLE 1. FRAGMENTATION EFFICIENCIES OF GLYCOSAMINOGLYCANS

GAG	Oxidant	Rate constant ($M^{-1} \cdot s^{-1}$)	Fragmentation efficiency	References	GAG	Oxidant	Rate constant ($M^{-1} \cdot s^{-1}$)	Fragmentation efficiency	References
Hep	•OH	2.2×10^8	8%	(8, 132, 133)	HA	•OH	4×10^8	32%	(8, 132, 133)
	$CO_3^{\bullet-}$	5×10^4	6%	(132, 133)		$CO_3^{\bullet-}$	3.5×10^4	11%	(132, 133)
	HOCl	$\sim 0.15^a$	<0.4%	(4)		HOCl	$\sim 0.01^a$	<0.4%	(4)
Hep-Cl	•OH	1.6×10^8	$\sim 100\%^b$	(132, 133)	HA-Cl	•OH	2.2×10^8	100% ^b	(132, 133)
	$CO_3^{\bullet-}$	8×10^4	29%	(132, 133)		$CO_3^{\bullet-}$	1.2×10^5	100%	(132, 133)
	HOCl	0	—	(4)		HOCl	3×10^{-2}	—	(4)

•NO₂ and O₂^{•-} do not directly induce GAG fragmentation and were therefore not included in the table.

^aCombined rate for both oxidation and substitution reactions.

^bCalculated from the increase in fragmentation efficiency following GAG substitution by HOCl, taking the extent of GAG substitution into account. See references for details.

•NO₂, nitrogen dioxide; •OH, hydroxyl radical; $CO_3^{\bullet-}$, carbonate radical anion; GAG, glycosaminoglycan; HA, hyaluronic acid; HA-Cl, hyaluronic acid chloramide; Hep, heparin; Hep-Cl, heparin chloramide; HOCl, hypochlorous acid; O₂^{•-}, superoxide anion.

gel electrophoresis (PAGE) (65). Using ionizing radiation to generate free radicals, it was suggested that $CO_3^{\bullet-}$ and •OH react in a site-specific manner to produce HA fragments in a ladder pattern. On Alcian blue/silver-stained PAGE gels, each band was separated from its neighboring bands by a distance equivalent to the molecular mass of the repeating HA disaccharide (65), thereby mimicking the action of the HA-degrading enzyme hyaluronidase. Due to the nonlinear relationship between fragment size and the Alcian blue/silver stain, no fragmentation efficiencies could be calculated (65). In the same study, EPR spin trapping experiments provided evidence for the formation of C-4 radicals on the GluA moiety with subsequent scission of the radical-containing GAG as a major route to HA fragmentation (65). In contrast to HA, heavily charged heparin, which serves as a model GAG for HS, proved particularly resistant to degradation by •OH and $CO_3^{\bullet-}$, exhibiting fragmentation efficiencies of only 8% and 6%, respectively (132) (Table 1).

The aforementioned ionizing radiation studies have unveiled two important issues regarding GAG oxidation by free radicals. The first entails the possible selectivity of attack by •OH and $CO_3^{\bullet-}$ that is evident from the ladder pattern observed in Alcian blue/silver-stained PAGE gels (65) and the absence thereof in Alcian blue-only stained gels (6, 132). This may be attributable to the difference in staining methods or to a reduced extent of degradation in the latter studies, which would make the ladder pattern difficult to detect. To explain the ladder-type fragmentation of GAGs, however, the reactions preceding the formation of C-4 radicals and subsequent GAG chain scission have to be equally likely for both •OH and $CO_3^{\bullet-}$, which is questionable given the difference in reactivity of the two species toward GAGs (6, 133). The second issue is the effect of GAG charge on the efficiency of fragmentation and the selectivity of radical attacks. The low fragmentation efficiencies for reactions between •OH and $CO_3^{\bullet-}$ and heparin may support either a highly selective attack or an effect of charge on the scission process after the formation of the GAG radical. Both phenomena may also apply (also see the Modification and Fragmentation of GAGs by Nonradical Halogen-Containing Oxidants section).

However, not all biologically relevant radicals induce GAG chain breaks. In a laser flash photolysis study (performed at pH=8.5), the reaction between O₂^{•-} and HA or heparin only showed O₂^{•-} dismutation, implying very low or no reactivity of O₂^{•-} toward these GAGs (109). Subsequent fragmentation studies confirmed the lack of reactivity and (direct) GAG fragmentation by O₂^{•-} (132). Similarly, •NO₂ generated *via* ionizing radiation did not induce fragmentation of HA, CS, or heparin as assessed by PAGE (65, 132). •NO₂ is a weak oxidant (28), and the rate constants for the reactions with GAGs are therefore expected to be low, particularly with respect to H-abstraction. It must be noted that reactions between •NO₂ and GAGs that proceed exclusively *via* non-fragmentation pathways cannot be excluded.

The reaction of ONOO⁻/ONOOH with HA has been studied using stopped-flow techniques combined with GPC/MALLS to measure changes in the HA molecular mass distribution. It was concluded that fragmentation was mediated by ONOOH-derived •OH, which accounted for 5% of the total ONOO⁻/ONOOH concentration. Alternatively, ONOOH may have directly fragmented HA (5). A later study demonstrated that both pre-synthesized ONOO⁻/ONOOH

and 3-morpholinodisodnonimine (SIN-1) (which generates peroxynitrite *in situ*) reacted with HA, CS, HS, and heparin and yielded a ladder pattern in PAGE experiments, particularly in case of HS and CS. This study also confirmed the participation of both $\bullet\text{OH}$ and ONOOH but not ONOO^- in the fragmentation process (65).

Modification and fragmentation of GAGs by nonradical halogen-containing oxidants. The mechanisms of GAG modification and fragmentation by HOCl are more complex than those described above since both substitution and redox reactions are involved. HOCl has a pK_a of 7.59 (86, 167) and is therefore (approximately) in equilibrium with its anion OCl^- at physiological pH. In earlier studies at $\text{pH}=7.4$, it was shown that the substitution of the GAG amino proton by a chlorine to form chloramines and chloramides constitutes the predominant reaction between HOCl/OCl^- and GAGs (118, 119, 121). However, HOCl and OCl^- are also capable of initiating oxidizing reactions, which could directly induce GAG fragmentation.

In a recent investigation, the reactions of HOCl/OCl^- with HA and heparin were studied as a function of pH ($\text{pH}=6.5\text{--}8.5$) (4). Spectral, chloramide yield, and kinetic measurements showed sharply contrasting behavior with increasing pH for heparin and HA. For HA, substitution was the dominant process for both HOCl and OCl^- , with chloramide yields of 100% across the entire pH range. For the negatively charged heparin, the chloramide yield was 94% at $\text{pH}=6.5$ but only 60% at $\text{pH}=8.5$. It was therefore proposed that the O- and N-sulfated groups in heparin hinder the substitution reaction, especially in case of OCl^- at higher pH, increasing the rate of oxidation. Additionally, at high $\text{HOCl}:\text{HA}$ ratios, it was shown that HOCl reacts with the formed HA chloramides but not with heparin chloramides. Despite the capacity to oxidize GAGs directly, the fragmentation efficiency of HA and heparin by HOCl/OCl^- was $<0.4\%$ (4).

Notwithstanding the limited induction of direct chain breaks, substitution at the amino groups by HOCl/OCl^- does affect GAG fragmentation. Because GAG chloramides are weak oxidizing agents, they are potential targets for reductants. In contrast to the absence of reactivity between the weak reducing agent $\text{O}_2^{\bullet-}$ [$E(\text{O}_2/\text{O}_2^{\bullet-}) = -0.33\text{ V}$] (99) and nonchlorinated GAGs (see the Oxidative Fragmentation of GAGs by Biologically Relevant Radicals and Nonradical Halogen-Lacking Oxidants section), EPR experiments demonstrated that thermally generated $\text{O}_2^{\bullet-}$ causes fragmentation of chlorinated HA following reduction of the chloramide (119). It was proposed that $\text{O}_2^{\bullet-}$ first transfers an electron to the chloramide, which results in the formation of a nitrogen-centered radical that subsequently undergoes intramolecular rearrangements to yield a C-2 radical on the GlcNAc moiety or a C-4 radical on the GluA moiety (117, 119) (Fig. 6). The C-2 and C-4 radicals quickly rearrange to either induce GAG chain scission or decompose *via* nonfragmentation routes [Figure 6, described in detail by Rees and Davies (117)]. In a later study, it was concluded that the fragmentation of HA- and HS chloramides by $\text{O}_2^{\bullet-}$ was mediated, at least in part, by catalytically active TMs that had been reduced by $\text{O}_2^{\bullet-}$ (117). Using laser flash photolysis, rate constants for the direct reduction of HA- and heparin chloramides by $\text{O}_2^{\bullet-}$ were found to be $k \approx 2.2\text{--}2.7 \times 10^3\text{ M}^{-1}\cdot\text{s}^{-1}$ (109), supporting the mechanism of $\text{O}_2^{\bullet-}$ -mediated GAG chloramide fragmenta-

tion proposed by Rees *et al.* (117, 119). The relative contribution of the indirect (TM-mediated) and the direct reaction of $\text{O}_2^{\bullet-}$ with GAGs likely depends on the pH, whereby both reactions contribute at neutral pH and the direct reaction becomes predominant at higher pH (109).

To identify the reaction products that are formed upon the reduction of GAG chloramides, pulse radiolysis was used to expose HA- and heparin chloramides to strongly reducing formate radicals and hydrated electrons (133). As described for $\text{O}_2^{\bullet-}$, the GAG chloramides were quickly converted to nitrogen-centered radicals by both formate radicals and hydrated electrons. The absorption spectra of the reaction products were identical for both GAGs and consistent with an initial attack at the N-Cl moiety, ultimately resulting in the formation of C-2 radicals on the GlcNAc ring (133). In contrast to earlier reports (117), there was no evidence for the formation of C-4 radicals on the adjacent GluA moiety following chloramide reduction by formate radicals or hydrated electrons (133).

In the same pulse radiolysis study (133), $\bullet\text{OH}$ was shown to react rapidly, presumably *via* H-abstraction, with GAG chloramides at rate constants of $k \approx 2.2 \times 10^8\text{ M}^{-1}\cdot\text{s}^{-1}$ (HA) and $k \approx 1.6 \times 10^8\text{ M}^{-1}\cdot\text{s}^{-1}$ (heparin). There was no evidence for a preference for the N-Cl group compared to other sites of attack. $\text{CO}_3^{\bullet-}$, which has a lower oxidizing potential than $\bullet\text{OH}$, reacted more slowly with the GAG chloramides than $\bullet\text{OH}$ ($k \approx 1.0 \times 10^5\text{ M}^{-1}\cdot\text{s}^{-1}$ for HA chloramides and $k \approx 8.0 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$ for heparin chloramides) but faster than with unsubstituted GAGs ($k \approx 3.5 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$ for HA and $k \approx 5.0 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$ for heparin). It was therefore postulated that the N-Cl group in substituted HA and substituted heparin constitutes a more attractive target for reducing species ($\text{O}_2^{\bullet-}$) as well as the oxidant $\text{CO}_3^{\bullet-}$ than the N-H group.

The effects of these findings on GAG fragmentation efficiencies were next investigated using ionizing radiation in combination with PAGE and GPC/MALLS (132). Despite the lack of selectivity, both heparin and HA chloramides were more efficiently fragmented by $\bullet\text{OH}$ than the unsubstituted parent molecules (132) (Table 1). This increase in efficiency is explained by the fact that N-Cl groups, although not a preferred target, give rise to (almost) 100% efficient GAG fragmentation when oxidized by $\bullet\text{OH}$ (Table 1). Similarly, increased fragmentation efficiencies were found when $\text{CO}_3^{\bullet-}$ reacted with heparin- and HA chloramides *versus* the unsubstituted parent compounds (132) (Table 1). $\text{CO}_3^{\bullet-}$ reacts preferentially with N-Cl groups (see above) and fragments HA and heparin chloramides at efficiencies of 100% and 29%, respectively (Table 1). Abstraction of the chlorine atom from N-Cl by $\text{CO}_3^{\bullet-}$ would result in the formation of a nitrogen-centered radical that would, at least in part, rearrange to a C-2 radical on the GlcNAc moiety or a C-4 radical on the GluA moiety, as proposed earlier (117) (Fig. 6). These radicals could induce GAG chain scission by reacting with O_2 to form peroxy radical intermediates, which decompose according to mechanisms described elsewhere (117, 118).

With respect to reducing species, hydrated electrons fragmented HA chloramide and heparin chloramide at an efficiency of 100% and 25%, respectively (132). Given the lower fragmentation efficiency for heparin chloramides, it was proposed that the charged sulfate groups in heparin alter

FIG. 6. Possible molecular fates of nitrogen-centered (amidyl) radicals that are formed upon oxidation or reduction of GAG chloramides. The nitrogen-centered radicals are short-lived and rearrange *via* intramolecular proton transfer to either a C-2 radical on the GlcNAc moiety (left pathway) or C-4 radicals on the GluA moiety (right pathway). Both radicals can rearrange *via* nonfragmentation pathways or induce GAG strand cleavage. The relative importance of each pathway is detailed in the Modification and Fragmentation of GAGs by Nonradical Halogen-Containing Oxidants section. The fate of the C-2 and C-4 radicals is also discussed in Refs. (117, 118, 132, 133). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the fate of the C-2 radical that is formed after chloramide reduction (117, 118, 133) toward nonfragmentation decomposition routes (132) (Fig. 6). The weaker reducing agent $O_2^{\bullet-}$ did not cause fragmentation of either HA or heparin chloramides, although reactivity had been shown earlier (109). It was postulated that $O_2^{\bullet-}$ reduces the chloramide to a nitrogen-centered radical that subsequently reacts with oxygen to produce a stable nitroxide species (shown tentatively in Figure 7).

In conclusion, it seems that HOCl/OCI⁻ can damage the GCX by forming GAG chloramides that become a site-specific target for $CO_3^{\bullet-}$ and $O_2^{\bullet-}$ and are more efficiently fragmented by both $CO_3^{\bullet-}$ and $\bullet OH$. The evidence presented here also shows that sulfated GAGs such as heparin and HS are more resistant to fragmentation by ROS/RNS than uncharged HA.

Sources of ROS and RNS

For GCX degradation to occur, ROS/RNS must be formed in the direct vicinity of the endothelial surface. Since the GCX is mainly degraded by $\bullet OH$, $CO_3^{\bullet-}$, and HOCl, the availability of

the template oxidants $O_2^{\bullet-}$ and $\bullet NO$ or the combined presence of H_2O_2 and MPO (Fig. 5) is necessary for ROS/RNS-induced GCX damage. In light of the shifting ROS/RNS production sites with increasing reperfusion time (see the ROS and RNS in Hepatic I/R Injury section), the earliest sources of GCX-afflicting ROS/RNS are likely to be ECs and KCs during the acute reperfusion phase. After the inflammatory response has been activated by KCs, ROS/RNS-producing leukocytes migrate to the liver and become stationary in the microvasculature, where they deliver a second oxidant/radical hit to the GCX during the chronic reperfusion phase.

Kupffer cells. Because the liver is mainly supplied with pathogen-rich blood *via* the portal tract, the hepatic microcirculation is replete with KCs that monitor the blood stream for signs of danger. Although innately programmed to detect microbes, KCs are also activated by endogenous danger signals (*i.e.*, DAMPs) that are released into the circulation during sterile inflammatory conditions, such as hepatic I/R injury (84, 148). Following activation, KCs assemble phagocyte NADPH oxidase (NOX2) and reduce oxygen to $O_2^{\bullet-}$ in order to kill and/or

FIG. 7. Proposed mechanism for the reaction of $O_2^{\bullet -}$ with GAG chloramides. Reduction of the chloramide initially yields a nitrogen-centered (amidyl) radical (top right) that subsequently reacts with O_2 to form a peroxy radical intermediate ($N-O-O^\bullet$, left center). Two GAG peroxy radicals subsequently react to ultimately form two stable GAG nitroxides (bottom) without inducing GAG chain scission. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

digest phagocytosed material (33, 76). The assembly of NOX2 is swift and relies on the phosphorylation-driven relocalization of pre-synthesized subunits (p40, p47^{phox}, p67^{phox}, and Rac) to the plasma membrane (48, 49, 131).

Although NOX2 only generates extracellular $O_2^{\bullet -}$ that is short-lived [estimated $t_{1/2} \sim 10^{-6}$ s (44)] and therefore unlikely to directly cause endothelial GCX degradation, the reactive derivatives of $O_2^{\bullet -}$ (Fig. 5) are detrimental to the GCX, particularly in the presence of $\bullet NO$. KCs upregulate inducible nitric oxide synthase (iNOS) during reperfusion (68), as a result of which $O_2^{\bullet -}$ and $\bullet NO$ are produced concurrently by the same cell. The long half-life [$t_{1/2} > 1$ s (107)] and considerable diffusion distance [200 μm (77)] allow $\bullet NO$ to reach the site of $O_2^{\bullet -}$ production. Inasmuch as $O_2^{\bullet -}$ is produced at a rate of 5.2 mM/s by NOX2 (in a phagosome) (166) and the reaction between $\bullet NO$ and $O_2^{\bullet -}$ is diffusion-controlled (53, 69), the ONOO⁻/ONOOH yield is high, as evidenced by the rapid (<2 h) rise in protein nitration following hepatic I/R injury in mice (87). More importantly, the estimated diffusion distance of ~ 50 μm (165) should enable KC-derived ONOO⁻/ONOOH to reach the GCX and initiate GCX oxidation after decaying to its derivative radicals $\bullet OH$ and/or $CO_3^{\bullet -}$ (Fig. 5). Any $O_2^{\bullet -}$ that escapes the reaction

with $\bullet NO$ dismutates spontaneously to H_2O_2 , which has an estimated diffusion distance of 1.5 mm (165) and a $t_{1/2}$ of $\sim 10^{-5}$ s (44), indicating that H_2O_2 is also capable to reach the GCX and participate in the reactions detailed in the Mechanisms of GCX Oxidation section.

Endothelial cells. During the acute reperfusion phase, ECs also produce ROS/RNS in the direct vicinity of the GCX. The predominant source of ROS/RNS in ECs during reperfusion is eNOS, which normally controls vascular tone by oxidizing L-arginine to L-citrulline and $\bullet NO$ in the presence of O_2 , NADPH, the coenzyme calmodulin, and the cofactor tetrahydrobiopterin (BH_4) (7). During oxidative/nitrosative conditions such as I/R, the functional 150 kDa eNOS dimer is destabilized (uncoupled), at which point the electrons derived from NADPH oxidation are transferred to O_2 instead of L-arginine, resulting in the production of $O_2^{\bullet -}$ at the expense of $\bullet NO$ (155).

The conversion of eNOS to a ROS-generating enzyme is instigated during ischemia, which induces an ischemia time-dependent depletion of the eNOS cofactor BH_4 (34). When the circulation is restored, eNOS uncoupling is enhanced by ONOO⁻/ONOOH, which oxidizes the catalytic zinc-thiolate

cluster of eNOS (176) and/or oxidizes BH_4 to dihydrobiopterin (BH_2) (23), both resulting in eNOS-mediated $\text{O}_2^{\bullet-}$ production. It was recently shown that MPO-derived oxidants such as HOCl also readily oxidize protein zinc-thiols, which could further augment eNOS uncoupling (25). Although the enzymatic reduction of BH_2 to BH_4 by dihydrofolate reductase (DHFR) normally restores eNOS dimerization (26, 27), the expression of DHFR can be suppressed by H_2O_2 (20) and exacerbate eNOS uncoupling during I/R. Corroboratively, optimizing eNOS function during reperfusion by BH_4 supplementation ameliorates hepatic I/R injury in mice (35).

Nonresident leukocytes. In the chronic phase of hepatic I/R injury, the influx, adhesion, and diapedesis of myeloid lineage cells results in a second major oxidative/nitrosative attack on the GCX. Of these leukocytes, neutrophils are most notorious for their involvement in the pathogenesis of hepatic I/R injury (55). During hepatic I/R-induced sterile inflammation, neutrophils tethered to the endothelium release their ROS/RNS arsenal into the extracellular space when concomitantly stimulated by cytokines and EC adhesion molecules (e.g., ICAM-1) (96, 97). The oxidizing potential of activated neutrophils exceeds that of KCs because neutrophils not only coexpress NOX2 and iNOS but also release MPO (95, 167). Due to its positive charge, MPO binds to anionic GAGs on the endothelial surface (9) where it catalyzes GCX decomposition reactions. MPO is a heme peroxidase that reacts with a wide range of substrates and therefore alters the combination of ROS/RNS that are produced. The most relevant reaction with respect to the GCX is the reaction between MPO and H_2O_2 , yielding HOCl/OCl^- . The latter is either directly responsible for GAG fragmentation or harms the GCX by reacting with $\text{O}_2^{\bullet-}$ to form $\bullet\text{OH}$ ($k \approx 6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) (114) (see the Mechanisms of GCX Oxidation section).

The ROS/RNS-specific contribution of MPO is complicated given the number of reactions that the enzyme is involved in. First, the reaction between H_2O_2 and MPO ($k \approx 2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) competes with the conversion of $\text{O}_2^{\bullet-}$ to H_2O_2 by MPO ($k \approx 2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$), as a result of which the steady-state concentration of $\text{O}_2^{\bullet-}$ decreases ~ 5 -fold (166). The formation of H_2O_2 from $\text{O}_2^{\bullet-}$ by MPO should favor the subsequent generation of HOCl from H_2O_2 , which in turn could occur at the expense of the ($\text{O}_2^{\bullet-}$ -dependent) production of $\text{ONOO}^-/\text{ONOOH}$ and derivative radicals ($\text{CO}_3^{\bullet-}$, $\bullet\text{OH}$).

It should be noted that this calculation was based on kinetic modeling of a neutrophil phagosome, which is a confined intracellular compartment that is optimized for high-yield bactericidal ROS production. These calculations may therefore deviate somewhat in a sterile inflammatory setting, such as hepatic I/R injury, where phagocytosis is less prominent. Second, it must be emphasized that $\bullet\text{NO}$ was excluded from the equation, which should influence the aforementioned reactions between $\text{O}_2^{\bullet-}$, H_2O_2 , and MPO considerably. The reaction between $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ [$k \approx 1 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ (53, 69)] not only consumes $\text{O}_2^{\bullet-}$ at an extremely high rate but also generates $\text{ONOO}^-/\text{ONOOH}$ that oxidizes MPO to what is referred to as a compound-II intermediate ($k \approx 6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) (39, 43). Compound-II is subsequently recycled to native MPO by electron donors

such as $\text{O}_2^{\bullet-}$ or organic substrates (e.g., tyrosine), with the latter reaction generating a radical intermediate that can result in, for example, tyrosine nitration or lipid peroxidation [discussed in Refs. (29, 167)]. Nevertheless, the exact interplay between $\text{O}_2^{\bullet-}$, $\bullet\text{NO}$, and MPO in modulating GCX degradation is currently elusive.

In addition to neutrophils, inflammatory (Ly6C^{hi}) monocytes also invade the reperfused mouse liver following I/R, where they contribute to ROS production to a similar extent as KCs and neutrophils (10). Like neutrophils, monocytes activate NOX2 and iNOS upon stimulation (52, 94) and excrete MPO (141, 142). Monocytes and neutrophils are therefore equally capable of degrading the GCX through the reactions detailed in the Mechanisms of GCX Oxidation section.

Hepatic GCX Degradation *In Vivo*

Hepatic GCX degradation following I/R in mice

In addition to the *in vivo* proof of concept regarding the presence of a functional hepatic GCX (see the Hepatic GCX: *In Vivo* Proof of Concept section), GCX degradation as a result of hepatic I/R injury was also assessed in mice. To that end, the release of HS into the circulation was measured as a surrogate marker for GCX degradation (Fig. 8), as has been performed previously (21, 102, 122). Mice that were

FIG. 8. HS is released into the circulation following hepatic I/R in mice. Male C57Bl/6J mice (8–12 weeks old) were subjected to 60 min of partial liver ischemia or a sham operation ($N = 5$ –6/group). Ethylenediaminetetraacetic acid-anticoagulated blood samples were collected after 1 or 6 h of reperfusion or 6 h after sham operation. Plasma HS levels were assessed using the General Heparan Sulfate ELISA kit from Amsbio (catalog no. E0623Ge; Milton, United Kingdom). Data are shown as mean $\pm$ standard deviation and *indicates $p < 0.025$ compared to the sham group. Statistical analysis was performed using the Kruskal–Wallis test and *post hoc* Mann–Whitney *U* tests with an adjusted significance level of 0.025 to correct for multiple testing. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

subjected to hepatic I/R injury exhibited a rapid increase in plasma HS, which peaked after 1 h of reperfusion and remained elevated after 6 h of reperfusion (Fig. 8). Although additional experiments are required to prove that the rise in plasma HS is due to GCX degradation, these data provide preliminary support for the potential link between GCX injury and hepatic I/R injury.

Hepatic GCX degradation following I/R in patients

To evaluate if the animal data reflect the clinical situation, serum HS levels were also assessed in patients that had undergone a major liver resection. During this type of operation, the decision whether or not to apply vascular inflow occlusion (*i.e.*, induce hepatic I/R injury) was made during the surgery based on the operative course (*e.g.*, extent of blood loss). A total of 18 patients were therefore intraoperatively assigned to either the I/R ($N=11$) or the control (CTRL, $N=7$) group. The patient characteristics are listed in Supplementary Table S1.

As in the mouse experiments, HS was already released into the circulation during the first hour after hepatic I/R injury in patients (Fig. 9C, D). However, a similar increase in circulating HS was found in patients who underwent a liver resection without I/R (Fig. 9A, B), suggesting that I/R is not

responsible for the rise in serum HS. In an earlier report that found a postoperative increase in circulating HS and syndecan-1 in patients who had undergone cardiopulmonary bypass or clamping of the aorta, thereby inducing cardiopulmonary or global ischemia, the extent of HS and syndecan-1 release also correlated poorly with the duration of ischemia (122). Although it is difficult to explain these findings at this point, it must be noted that the rise in serum HS could contribute to postoperative liver injury *via* the proinflammatory mechanisms described in the Proinflammatory Consequences of GCX Degradation section, irrespective of the underlying cause.

There are several factors that should be taken into account when interpreting the current results. Hepatic I/R injury in patients inherently suffers from vast interindividual variations. As opposed to the HS study in mice, the limited experimental standardization is most evident from the lack of correlation between the duration of ischemia and the peak postoperative value of the hepatocellular injury marker alanine aminotransferase (ALT, Fig. 9F). The same poor correlation was observed when comparing the peak fold increase in serum HS to the duration of ischemia or the peak postoperative ALT values (Fig. 9G, H). The data could also be distorted by the heterogeneous composition of the study

FIG. 9. Circulating HS levels in patients who had undergone a major liver resection. Serum samples were obtained from all patients ($N=18$) before liver resection and after 1 and 6 h of reperfusion (I/R group) or 1 and 6 h after resection (CTRL group). Patient characteristics are included in Supplementary Table S1. Serum HS content was assessed by enzyme-linked immunosorbent assay (Fig. 6) and is reported as mean $\pm$ SEM. The results were normalized to plasma protein content, determined with the Pierce Total Protein Assay Kit (Rockford, IL), to correct for hemodilution. The red squares indicate the mean per time point. Intragroup differences were assessed using the Friedman test for repeated measurements in combination with Wilcoxon signed-rank *post hoc* testing ($t=1$ and $t=6$ vs. $t=0$). Because the significance level was adjusted to correct for multiple testing, * and # indicate $p<0.025$ compared to $t=0$ in the CTRL and I/R group, respectively. In the CTRL group (A), HS concentrations were 0.98 ± 0.48 ng/mg protein at baseline, increased to 2.13 ± 0.86 ng/mg protein 1 h after liver resection, and decreased to 0.43 ± 0.15 ng/mg protein 6 h after liver resection. In the I/R group (C), the HS concentration was 1.85 ± 0.79 ng/mg protein at baseline, increased to 2.88 ± 1.09 ng/mg protein after 1 h of reperfusion, and dropped to 0.30 ± 0.078 ng/mg protein after 6 h of reperfusion. The same data are plotted as fold increase compared to baseline in (B, D, E), yielding a peak mean fold increase of 3.55 ± 0.94 1 h after liver resection in the CTRL group and a mean $\pm$ SEM peak fold increase of 5.28 ± 2.96 after 1 h of reperfusion in the I/R group. (F, G) Show correlation plots and linear regression lines of the cumulative duration of ischemia plotted against the peak in postoperative ALT [a liver damage marker, (F)] and postoperative peak fold increase in circulating HS (G). (H) Shows the correlation between the postoperative peak ALT and peak HS fold increase. ALT, alanine aminotransferase; CTRL, control; SEM, standard error of the mean. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

cohort, in particular the differences in underlying hepato-pathologies. Most patients who undergo a liver resection have a primary or secondary hepatic malignancy (83.3% of the study participants, see Supplementary Table S1), which is associated with increased degradation of extracellular matrix components, such as HS and syndecan-1 (123, 172). Consequently, factors related to tumor biology may have skewed the data. Also, the resection itself may cause liver parenchyma constituents, including GAGs, to become blood-borne. Inasmuch as the resections were not standardized, the procedure itself may have differentially affected circulating HS levels after the surgery. Due to these factors and the small group sizes, it is difficult to draw definitive conclusions from the current data. Additional experiments are therefore under way to investigate the factors that drive the increase in circulating HS following major liver resection.

Proinflammatory Consequences of GCX Degradation

Damage to the GCX exacerbates hepatic I/R injury *via* two distinct routes. On the one hand, the liver is stripped from the protective functions that the GCX normally imparts (see the GCX Function section), which leads to increased oxidative/nitrosative stress *via*, for example, the loss of GCX-bound ecSOD (75) (directly) and exposure of endothelial leukocyte receptors such as ICAM-1 (129) (indirectly). On the other hand, circulating GAG fragments have been characterized as DAMPs capable of amplifying the hepatic I/R-induced immune response by directly activating leukocytes and ECs.

The proinflammatory actions of HA differ based on the size of the HA fragment. Low molecular mass (<30 kDa) HA stimulates toll-like receptors (TLRs) on the surface of macrophages [TLR-2 and/or TLR-4 (128, 144)], dendritic cells [TLR-4 (145, 146)], and ECs [TLR-4 (143)]. In doing so, small HA fragments trigger the production of I/R-pertinent (151) cytokines, such as tumor necrosis factor alpha (TNF- α). HA fragments that are >30 kDa do not activate cell surface receptors but are endocytosed *via* CD44, incorporated into lysosomes, and enzymatically digested to a size of <20 kDa. The digested fragments subsequently induce the assembly of a proinflammatory protein complex known as the NALP3 inflammasome (3) that ultimately governs the release of the proinflammatory cytokines interleukin (IL)-1 β and IL-18 (170). Like HA, HS also tethers to TLR-4 on the surface of macrophages and dendritic cells, leading to the mobilization of the proinflammatory transcription factor nuclear factor- κ B and the production of proinflammatory mediators, such as TNF- α and IL-1 β (58, 73). Corroboratively, TLR-4-deficient mice were found to be resistant to the intravenous administration of an otherwise lethal dose of HS (5 mg/mL) (59). These results, however, have mostly been generated using enzymatically digested GAGs. Considering that the proinflammatory effects of DAMPs such as high-mobility group box 1 can be switched on or off by redox modifications (61, 156, 171), it remains to be shown whether oxidatively fragmented GAGs have the same immunogenic potential as their enzymatically digested counterparts.

Heparanase

The premise that ROS/RNS are exclusively injurious *via* direct oxidation has been superseded by a more refined paradigm, which stipulates that low levels of ROS/RNS rather

serve as signaling molecules [*e.g.*, through protein thiol oxidation (92)] and mainly do (direct) harm when generated in excess. In this light, ROS/RNS could pose an indirect threat to the GCX by triggering the activation and release of the HS-degrading enzyme heparanase (30). Heparanase is an endo- β -D-glucuronidase that is stored in the lysosomes of ECs, leukocytes, and platelets (12) as an inactive precursor, which is proteolytically processed by cathepsins (1, 130) to a catalytically active heterodimer comprising 50 and 8 kDa subunits (54, 161). When activated, heparanase cleaves the glycosidic bond between the GlcNAc and GluA/IduA rings of HS, albeit there is currently no consensus on the exact composition (*e.g.*, sulfation pattern) of the HS motif that is recognized by heparanase (104, 110).

It has recently been shown that heparanase sheds HS from the GCX of pulmonary microvessels in a mouse model of lipopolysaccharide (LPS)-induced sepsis (129). Following the systemic administration of LPS, the functional GCX thickness measured by intravital fluorescence microscopy quickly (<30 min) decreased by $\sim$ 3-fold, leading to a vast increase in neutrophil accumulation and vascular permeability (129). These effects could be reproduced by administering TNF- α instead of LPS and were diminished in LPS-primed animals genetically deficient in either heparanase or the TNF- α receptor-1 (129), indicating that TNF- α -induced heparanase activation accounted for the disintegration of the pulmonary GCX. These findings were substantiated by a rise in heparanase activity in plasma of severely septic patients and enhanced heparanase expression in lung biopsies of septic patients who concomitantly suffered from acute lung injury (129).

Although the aforementioned mechanism of GCX injury applies to (infectious) pneumosepsis, several other routes to heparanase activation could be equally relevant in the context of hepatic I/R injury. First, ischemia (*i.e.*, hypoxia) *per se* could affect the expression and/or release of heparanase, probably owing to the established involvement of heparanase in (tumor) angiogenesis (62, 123). Transcriptional upregulation of heparanase was documented in HEK293 cells that were cultured for 24 h in a hypoxic (1% O₂) environment (126). In a cell-free system, human platelet-derived heparanase displayed an increase in activity following 8 h of hypoxia (1% O₂), which was corroborated by treating heparanase with the reducing agent β -mercaptoethanol, suggesting direct redox activation of the enzyme. Admittedly, the duration of ischemia in the cited studies exceeds the ischemic period typically used in hepatic I/R injury, which rarely is longer than 60 min (in patients) to 90 min (in mice). There is, however, little known about the response of heparanase to hypoxia *in vivo*, meaning that it remains to be shown how heparanase responds to ischemia in a clinically more representative setting.

In addition to hypoxia, the pro-oxidative state during early reperfusion could also contribute to heparanase-mediated GCX degradation. Human brain microvascular ECs cultured in the presence of H₂O₂ (1–20 μ M) not only upregulated heparanase at a transcriptional level but also released heparanase into the culture medium (115). Corroboratively, the release of heparanase by these cells, when exposed to oxidative stress-inducing concentrations of glucose, could be prevented by the treatment with the antioxidant glutathione or the glutathione precursor *N*-acetylcysteine (115). In both

scenarios, the antioxidant treatment increased cell surface HS expression as a result of decreased heparanase activity (115). Similarly, treatment with the antioxidant dimethylthiourea (41) suppressed heparanase expression in a rat model of doxorubicin-induced kidney injury, thereby preserving cell surface HS content (74).

The third hallmark event of I/R injury that is linked to heparanase activation is the release of DAMPs. Extracellular purines (ATP and its di-adenosine diphosphate [ADP] and adenosine monophosphate derivatives) are established DAMPs (15, 84) and have been detected extracellularly in experimental (15, 47) as well as clinical (47) liver I/R. With respect to heparanase, the treatment of HEK293 cells with either ATP or ADP resulted in a rapid (<60 min) release of catalytically active heparanase, an effect that was dependent on the concomitant mobilization of protein kinase C and that could be inhibited by blocking the purinergic cell surface receptor P2Y1 (130).

The last component of hepatic I/R injury that is related to heparanase is the activation of the coagulation cascade, as is reflected by the massive accumulation of platelets (67) and formation of microthrombi (105) in the I/R-subjected murine liver. During clot formation, activated platelets release heparanase into the circulation (169), which directly threatens the GCX. Because the GCX also harbors anticoagulant proteins, such as antithrombin-III (21) and tissue factor pathway inhibitor (106), the release of heparanase by platelets could propagate the coagulation cascade and promote thrombus formation through the loss of GCX-bound anticoagulant proteins.

In conclusion, heparanase has been recently identified as a major threat to the GCX in pulmonary sepsis. However, four aspects of hepatic I/R injury—hypoxia, oxidative stress, DAMP release, and thrombosis—also trigger heparanase release. Notably, all these events could contribute to GCX degradation during sterile inflammation.

Conclusions

With respect to critical care patients, the significance of the GCX has been firmly gripped by anesthesiologists, who consider the GCX as a promising therapeutic target to, for example, maintain an adequate fluid balance in critically ill patients (32, 168). The GCX may be equally relevant to (liver) surgeons (19, 122) insofar as preserving the GCX or inhibiting the proinflammatory signaling by GCX degradation products could alleviate I/R injury and thereby reduce the incidence of postoperative liver failure and corollary high mortality rates (51). Preliminary experiments indicate that hepatic sinusoids bear a functional GCX that roughly spans 0.2 μ m from the endothelial surface. Following hepatic I/R injury in both mice and patients, the plasma concentration of the GCX component HS increases, alluding to surgery-induced damage to the GCX. Patients who underwent a liver resection without I/R injury, however, showed a similar increase in circulating HS. The mechanisms underlying surgery-induced injury to the GCX therefore remain ill-defined and warrant further *in vivo* investigation, particularly with respect to the contribution of different ROS/RNS intermediates to GCX degradation, the immunogenic potential of the shed GCX fragments, and the role of heparanase in I/R injury.

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Abbreviations Used

ADP = adenosine diphosphate
ALT = alanine aminotransferase
ASA = American Society of Anesthesiologists
ATP = adenosine triphosphate
BH₂ = dihydrobiopterin
BH₄ = tetrahydrobiopterin
CO₂ = carbon dioxide
CO₃^{•-} = carbonate radical anion
CS = chondroitin sulfate
CTRL = control
DAMP = damage-associated molecular pattern
DHFR = dihydrofolate reductase
EC = endothelial cell
ecSOD = extracellular superoxide dismutase
eNOS = endothelial nitric oxide synthase
EPR = electron paramagnetic resonance
FITC = fluorescein isothiocyanate
GAG = glycosaminoglycan
Gal = galactose
GalNAc = N-acetylgalactosamine
GCX = glycocalyx
GlcNAc = N-acetylglucosamine
GluA = glucuronic acid
GPC = gel permeation chromatography
GTP = guanosine triphosphate
H₂O₂ = hydrogen peroxide
HA = hyaluronic acid
H-abstraction = hydrogen abstraction
HA-Cl = hyaluronic acid chloramide
Hep = heparin
Hep-Cl = heparin chloramide
HOCl = hypochlorous acid
HS = heparan sulfate
I/R = ischemia/reperfusion
ICAM-1 = intercellular adhesion molecule 1
IduA = iduronic acid
IL = interleukin
iNOS = inducible nitric oxide synthase
KC = Kupffer cell
LPS = lipopolysaccharide
MALLS = multiangle laser light scattering
MPO = myeloperoxidase
N-Cl = chloramide
NDD = nondescanned detector
•NO = nitric oxide
•NO₂ = nitrogen dioxide

Abbreviations Used (Cont.)

NOX2 = phagocyte NADPH oxidase
O₂ = oxygen
O₂^{•-} = superoxide anion
OCl⁻ = hypochlorite anion
•OH = hydroxyl radical
ONOO⁻ = peroxynitrite anion
ONOO⁻/ONOOH = peroxynitrite
ONOOH = peroxynitrous acid
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate-buffered saline
PG = proteoglycan

RNS = reactive nitrogen species
ROI = region of interest
ROS = reactive oxygen species
SEC = sinusoidal endothelial cell
SEM = standard error of the mean
SIN-1 = 3-morpholinodisodnonimine
SOD = superoxide dismutase
TLR = Toll-like receptor
TM = transition metal
TNF- α = tumor necrosis factor alpha
XOR = xanthine oxidoreductase
Xyl = xylose