Dextran sulfate modulates MAP kinase signaling and reduces endothelial injury in a rat aortic clamping model

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Objective: Mitogen-activated protein kinases (MAPKs), including JNK, p38, and ERK1/2, noticeably influence ischemia/reperfusion injury (IRI). The complement inhibitor dextran sulfate (DXS) associates with damaged endothelium denuded of its heparan sulfate proteoglycan (HSPG) layer. Other glycosaminoglycan analogs are known to influence MAPK signaling. Hypothetically therefore, targeted intravascular cytoprotection by DXS may function in part through influencing MAPK activation to reduce IRI-induced damage of the vasculature.

Methods: IRI of the infrarenal aorta of male Wistar rats was induced by 90 minutes clamping followed by 120 minutes reperfusion. DXS (5 mg/mL) or physiologic saline (NaCl controls) was infused locally into the ischemic aortic segment immediately prior to reperfusion. Ninety minutes ischemia-only and heparinase infusion (maximal damage) experiments, as well as native rat aorta, served as controls. Aortas were excised following termination of the experiments for further analysis.

Results: DXS significantly inhibited IRI-induced JNK and ERK1/2 activation (P = .043; P = .005) without influencing the p38 pathway (P = .110). Reduced aortic injury, with significant inhibition of apoptosis (P = .032 for DXS vs NaCl), correlated with decreased nuclear factor κB translocation within the aortic wall. DXS treatment clearly reduced C1q, C4b/c, C3b/c, and C9 complement deposition, whilst preserving endothelial cell integrity and reducing reperfusion-induced HSPG shedding. Protection was associated with binding of fluorescein labeled DXS to ischemicly damaged tissue.

Conclusions: Local application of DXS into ischemic vasculature immediately prior to reperfusion reduces complement deposition and preserves endothelial integrity, partially through modulating activation of MAPKs and may offer a new approach to tackle IRI in vascular surgical procedures. (J Vasc Surg 2009;50:161-70.)

Clinical Relevance: The purpose of the present study was to determine the role of dextran sulfate (DXS), a glycosaminoglycan analog and complement inhibitor, in modulating intracellular MAPK signaling pathways, reducing complement activation and ultimately attenuating ischemia/reperfusion injury (IRI) in a rat aortic-clamping model, in part a surrogate model to study the microvasculature. The study shows a role for DXS in ameliorating endothelial injury by reducing IRI-mediated damage and intravascular, local inflammation in the affected aortic segment. DXS may be envisaged as an endothelial protectant in vascular injury, such as occurs during vascular surgical procedures.

Mitogen-activated protein kinases (MAPK), comprising extracellular signal-regulated kinases (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38-MAPK, are a multi-gene family of serine/threonine protein kinases, which respond to a wide variety of stimuli, including environmental stresses and growth factors.1 Several studies have demonstrated a role for the MAPK family in ischemia/reperfusion injury (IRI). Activation of stress-induced JNK as well as p38 MAPK occurs upon ischemia and ensuing reperfusion,2 significantly contributing to target organ injury, and inhibition of the JNK and p38 MAPK pathways protects the ischemic and reperfused heart.3 Conversely, the ERK1/2 pathway has been shown to be protective.4 However, the situation is complex; a dynamic balance of the activities of these kinases may prove critical in deciding the outcome post-IRI. Endothelial cell (EC) activation is central to IRI. Disruption of the surface-protective endothelial glycocalyx layer,5 upregulation of endothelial adhesion molecules,6 as well as loss of anticoagulant properties7 promote a proinflammatory and procoagulant environment. Furthermore, activation and local upregulation of complement proteins8 plays a pivotal role in mediating tissue damage.

Complement-mediated induction of MAPK pathways, including ERK1/2 and JNK, has been described in mitogenic signaling in a variety of cells9 as well as chemotaxis in neutrophils through C5a receptor signaling.10 Low molecular weight dextran sulfate (DXS, MW 5000), a glycosaminoglycan analog, is a well-typed inhibitor of complement and coagulation pathways11,12 and

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associates with EC denuded of their glyocalyx heparan sulfate proteoglycans (HSPG).\textsuperscript{13} We have observed that DXS not only localizes to the cell surface but is also found intracellularly and inhibits phosphorylation of IκB-α and activation of nuclear factor κB.\textsuperscript{14} DXS may therefore influence intracellular signaling pathways. We postulated that, similar to glycosaminoglycans, DXS might also modulate MAPK signaling. The aim of this study therefore was to investigate aortic ischemic endothelial damage in a rat model and whether association of DXS with damaged endothelium and its known cytoprotective and complement-inhibitory effects correlate with modulation of the MAPK pathways.

This model may be of particular interest in vascular surgery, to study larger vessels, including the aorta, as well as surrogate for the (micro-) vasculature of the target organ(s), which may be subjected to longer periods of ischemic assault during, amongst others, complex surgical (revascularization) procedures.

**MATERIALS AND METHODS**

**Materials.** Low molecular weight DXS (sodium salt, MW 5000) was purchased from Sigma Chemical Company (St Louis, Mo). Fluorescein-labeled DXS (DXS-Fluo) was produced using fluorescein cadaverine (Molecular Probes Europe, Leiden, The Netherlands) as described earlier.\textsuperscript{13} It was verified that DXS-Fluo essentially had identical biochemical properties as the unlabeled substance.

**Experimental procedure.** The experimental protocol was approved by the veterinary office of the Canton of Bern, Switzerland. Care and use of animals in the present study was in compliance with national (Federal Law no. 455 on the protection of animals, 2005) as well as international guidelines. (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, revised 1996).

A total of 80 male Wistar rats (270 ± 65 g) were anesthetized by isoflurane/oxygen, through a semi-closed circuit inhalation system; spontaneous breathing was monitored and maintained throughout. The animals were placed on a heating pad and body temperature controlled to 37 ± 1°C. Prewarmed physiologic saline was administered intraperitoneally during the experiment, adapted to body weight and eventual blood loss from the aortic incision site (2.5 mg/kg/h + approximated volume of blood loss).

A summary of the experimental design, detailed below, is given in Fig 1.

Following a midline abdominal incision, the infrarenal aorta was mobilized as described by Neil et al.\textsuperscript{15} Although in a majority of the animals the clamped segment did not include lumbar arteries, where present, they were cauterized to ensure complete isolation of the aortic segment. After preparation, the aorta was clamped just distally of the renal arteries and carefully flushed with 1 mL of physiologic saline to remove any traces of blood. A second clamp was placed approximately 1 cm distally of the first clamp, proximally of the aortic bifurcation. After 90 minutes of ischemia, 200 μL physiologic saline (NaCl, controls, n = 20) or 200 μL of 5 mg/mL of DXS in NaCl (n = 20) were infused into the lumen of the ischemic aortic segment. Five minutes later, first the distal, then the proximal clamp were released and the aortic segment was reperfused for 120 minutes. The dose of DXS (<4 mg/kg body weight) was chosen from experience with previous experiments where in vivo dosages between 8 mg/kg up to 25 mg/kg were well tolerated, nontoxic and effectively without unwanted side effects.\textsuperscript{16,17}

Fifteen ischemia-only control experiments were performed in which the animals were sacrificed and the aortas excised after 90 minutes of clamping without reperfusion.

In a further 15 experiments, DXS-Fluo was used instead of the normal, unlabeled substances. These experiments were done in order to evaluate the binding of DXS to the endothelium and aortic wall. At the end of the experiments, the rats were sacrificed and the aortas were harvested for further evaluation.

**Heparinase III treatment.** With the hypothesis that endothelial HSPG release may play an important role in IRI and that this release prompts DXS binding, rats were subjected to aortic clamping for 10 minutes, during which 100 μL heparinase III (1 U/mL, Heparinase III from Flavobacterium heparinum; E.C.4.2.2.8), an enzyme selective for heparan sulfate within the glyocalyx, Fluka Chemie, Buchs, Switzerland) was injected into the aortic lumen (n = 6). Controls included 10 minutes incubation with heparinase III-buffer only (n = 4). Following incubation with heparinase III or buffer, the aortas were either excised immediately or reperfusion was induced. In further experiments, 200 μL DXS-Fluo was injected into the aortic segment after heparinase treatment, followed by reperfusion. All rats were kept under general anesthesia throughout the experiments, including the reperfusion phase.

**Immunostaining.** Rat aortic rings were retrieved (ischemic segment, nonischemic control segment), rinsed carefully with saline, embedded in Tissue-Tek OCT compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), and stored at −80°C until further analysis. Five μm sections were air-dried, acetone fixed, hydrated and labeled using a two-step indirect immunofluorescence...
Fig 2. Immunofluorescence staining for complement C1q, C4b/c, C3b/c, and C9 on rat aortic tissue. Essentially no or minimal complement staining following ischemia-only (I-only). Complement activation and deposition after 90 minutes ischemia /120 minutes reperfusion following NaCl administration as well as after infusion of heparinase (Hep III)/120 minutes reperfusion. Dextran sulfate (DXS) clearly reduces complement deposition. Scale bar represents 100μm. All images are representative of at least 4 scored sections per experiment. Grading (average score ± standard deviation); where 0: no staining, 1: minimal focal or diffuse staining, 2: moderately strong focal or diffuse staining, 3: extensive focal or diffuse staining.

Fig 3. Quantification of fluorescence intensity for complement staining/deposition of C1q, C4b/c, C3b/c, and C9. Histograms show a representative image of staining intensity (fluorescence, x-axis) from one of at least 4 individual experiments. Shift of the curve towards the right indicates increased intensity and thus increased complement deposition.
technique or immunohistochemical approach. The following antibodies were used: rabbit antisera specific for human C1q, C3b/c, and C4b/c (Dako, Glostrup, Denmark), rabbit anti-rat C9 (kind gift of Prof. Paul Morgan, Cardiff, UK), monoclonal mouse anti-human heparan sulfate (Seikagaku, Tokyo, Japan), rabbit antiserum specific for human von Willebrand Factor (vWF, Dako), and rabbit anti-human tissue factor (TF; American Diagnostica Inc, Stamford, Conn). Cross-reactivity with the respective rat antigens was verified for all used antibodies and antisera.

Secondary antibodies used were goat anti-rabbit IgG(F(ab')2)-FITC (Southern Biotechnology Associated, Birmingham, Ala) sheep anti-rabbit (F(ab')2)-Cy3 (Sigma), and rabbit anti-mouse (Ig)-FITC (Dako). Staining for phospho-JNK and phospho-ERK1/2 was performed with polyclonal antibodies specific for phosphorylated JNK and ERK1/2 respectively (Cell Signaling; BioConcept, Allschwil, Switzerland) followed by secondary labeled polymer using the Envision HRP Kit and diamino-benzidine chromogen kit (both Dako). After washing, the slides were counterstained with hematoxylin. All slides were mounted with SlowFade Light Antifade Kit (Molecular Probes Europe, Leiden, The Netherlands).

Nuclear factor (NF) κB was visualized using a rabbit anti-NF κB p65 (Abcam, Cambridge, Mass) antibody at 1:50 dilution, followed by secondary antibody (Dako) and counterstaining with hematoxylin.

Images were acquired with a fluorescence microscope (Nikon Eclipse TE2000-U; Nikon Corporation, Tokyo, Japan) and digital camera (Nikon DXM1200F). Images were captured with identical exposure times and settings in each experiment. For quantification of fluorescence intensity, Image J software (http://rsb.info.nih.gov/ij/) was used, in addition to grading of four samples per experiment with respect to staining intensity (as a further control to quantify staining): 0: essentially no staining; 1: minimal focal or diffuse staining; 2: moderately strong focal or diffuse staining; and 3: extensive diffuse staining. Sections were prepared such that grading was performed in a blinded manner by two independent observers.

For NF κB staining six random fields were chosen and nuclei staining positive for NF κB were counted and divided by the total number of nuclei identified in the field. Image J software was used for quantification of staining.

TUNEL staining for apoptosis. Apoptosis within the aortic wall was detected on serial 5 μm thick cryosections in high-power fields by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) according to the manufacturer’s instructions (In Situ Death Cell Kit; Roche Diagnostics, Basel, Switzerland). As a positive control, slides were incubated with DNase1 (Sigma, Chemical Co, St Louis, Mo) to induce DNA strand breaks. For every TUNEL stained section, a subsequent section was stained with hematoxylin-eosin to visualize total number of nuclei. Apoptosis was expressed by normalizing the results to the total number of nuclei per high-power field. Four high power fields per experiment and section were analyzed.

Western blotting for MAP kinases. Aortic segments were snap-frozen in liquid nitrogen and kept at −80°C until further processing for western blotting. Cell extracts were prepared by lysing cells from the aortic tissue samples in extraction buffer as described previously.18 Extracts of 40 μg were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to an Immobilon-P membrane (Millipore, Billerica, Mass). Following blocking with 5% skim milk, the membrane was incubated overnight at 4°C with the corresponding primary
antibody (polyclonal antibodies raised in rabbits for detection of JNK, ERK1/2, p38 MAPK and their respective phosphorylated counterparts; all Cell Signaling, BioConcept). This was followed by washing and incubation with an appropriate anti-rabbit alkaline phosphatase-conjugated secondary antibody. Signal was detected by Western Blue (Promega, Madison, Wis) stabilized substrate. Quantification of the signals was performed with NIH Image 1.62 software.

Statistics. Differences between as well as within the experimental groups were analyzed by one-way-analysis of variance (ANOVA). Post-hoc analysis was done using Bonferroni adjustments. Differences were considered to be significant with a P value of less than .05. Data, unless otherwise specified, are presented as average ± standard deviation in text and figures.

RESULTS

Tissue complement deposition. DXS treatment markedly reduced complement deposition after IRI compared with the NaCl control group: C1q (mean grading...
score; 1.0 ± 0.7 vs 2.1 ± 0.8), C3b/c (1.0 ± 0.7 vs 2.3 ± 0.7) and C9 (0.9 ± 0.7 vs 2.1 ± 0.8). This reduction was evident in stained tissue sections (Fig 2), quantitatively represented as histograms following analysis with image J software (Fig 3). Minimal complement deposition on the endothelial surface was detected after ischemia-only (mean grading score: C1q 0.4 ± 0.5; C4c 0.4 ± 0.5; C3c 0.8 ± 0.7; C9 0.3 ± 0.5). A similar, though more extensive staining pattern of complement deposition was observed after 10 minutes heparinase treatment followed by reperfusion compared with the NaCl ischemia/reperfusion control group. No complement deposition was detected upon heparinase administration without reperfusion or heparinase buffer only (not shown).

**Endothelial vWF and HSPG expression.** Expression of vWF was essentially maintained and detected circumferentially in all experimental settings. NaCl controls showed reduced staining intensity and, in certain experiments, focal absence of positivity, indicating partial endothelial denudation (Fig 4). Intensity of HSPG staining inversely correlated with complement deposition in all groups. Maximal HSPG staining (endothelial and extracellular matrix/cellular membranes within the vessel wall) was observed in native control aortas. HSPG staining was slightly decreased after 90 minutes ischemia only. A marked decrease or focal absence of HSPG was noted following 120 minutes reperfusion in NaCl controls. In contrast, HSPG intensity and distribution pattern was maintained at ischemia-only levels following DXS administration. Heparinase treatment almost entirely abolished HSPG positivity, whereas heparinase buffer had no effect (not shown).

**Tissue factor expression.** Compared with normal controls, tissue factor expression markedly increased in the NaCl group at the end of the 120-minute reperfusion phase compared with aortas from DXS-treated animals and ischemia-only controls (Fig 5).

**Binding of fluorescein-labeled dextran sulfate (DXS-Fluo).** DXS-Fluo binding was only detected in aortas subjected to ischemia or heparinase treatment and was not found on native aorta or after heparinase buffer alone (Fig 6, A). No DXS-Fluo binding was observed in other inspected organs (lungs, liver, kidney, spleen; not shown).

**TUNEL staining.** Compared with normal controls and aortas from ischemia-only experiments, noticeably more TUNEL positive nuclei were observed in the NaCl group within the aortic wall (Fig 6, B). DXS treatment significantly reduced TUNEL positivity within the aortic wall.
wall (TUNEL positive nuclei expressed as percentage of total nuclei per high power field: 8.5% ± 3.8% for DXS vs 21.4% ± 10.7% for NaCl, P = .032).

**p38 MAPK: Western blot.** p38 MAPK phosphorylation was significantly increased in the NaCl controls (P = .022, Fig 7, A) and heparinase III treated animals (P = .007, not shown) compared with normal aorta. Neither ischemia-only nor DXS treatment significantly affected p38 activation (DXS: P = .110; ischemia only: P = .440 vs normal).

**ERK1/2: Western blot and staining.** ERK1/2 phosphorylation was significantly increased in all experimental groups compared with normal aorta (P = .004 for ischemia-only, P = .0003 for NaCl controls, P = .0001 for DXS, Fig 7, B). ERK1/2 phosphorylation was not significantly higher in NaCl controls compared with ischemia-only experiments (P = .043). DXS treatment significantly reduced ERK1/2 activation compared with NaCl controls (P = .005). Immunohistochemical staining for activated ERK1/2, using an antibody specific for phosphorylated ERK1/2, confirmed increased levels in the NaCl controls compared with the DXS treated animals.

**JNK: Western blot and staining.** JNK phosphorylation was significantly increased in all experimental groups compared with normal aorta (P = .004 for ischemia-only, P = .0009 for NaCl controls, P = .0003 for DXS, Fig 7, C). DXS administration prior to reperfusion significantly inhibited JNK phosphorylation compared with NaCl controls (P = .043), down to levels of ischemia-only. Immunohistochemical staining for activated JNK, using an antibody specific for phosphorylated JNK, confirmed homogenously increased levels in the NaCl controls compared with the DXS treated animals.

**NFκB staining.** Immunohistochemistry revealed that the degree of IRI and complement activation correlated with the extent of NFκB nuclear translocation in the aortic wall. Minimal nuclear NFκB was observed in native aortas (Fig 8). However, there was substantial nuclear NFκB localization in the control NaCl treated aortas, with evidently less translocation following DXS administration.
DISCUSSION

Ischemia-induced damage, not only to the end organ but also to the vasculature itself, is of importance, particularly in vascular surgery that may necessitate temporary vascular clamping. The ensuing reperfusion may lead to substantial endothelial damage with HSPG release and denudation, promoting activation of complement and coagulation and resulting in potentially extensive damage not only to the end organ but also to the vasculature itself; whether it is the microvasculature within the end organ or the clamped (and at least partially ischemic) “conduit” artery or segments thereof. Indeed, although the thickness and composition of the endothelial glyocalyx differs according to type and size of the vessel, the response of the endothelium in particular and vasculature in general to (ischemia/reperfusion) injury involves the release of HSPG.

In a recent study by Rehm et al, it was shown for the first time in humans that significant shedding of the endothelial glyocalyx occurs in patients undergoing major vascular surgery with global and regional ischemia, including surgery for infrarenal aortic aneurysm.

Numerous aortic clamping models concentrate on end-organ injury. Whilst the presented model inevitably also affects the end organ, ie, hind limbs, with release of proinflammatory mediators, it chiefly also allows for more detailed immunohistochemical and biochemical studies of a vessel more amenable to such investigation than the corresponding microvasculature. By nature of the rather straightforward anatomy and surgical technique, the herein presented model essentially ensures quite standardized complete bilateral hind limb ischemia. This will induce a certain generalized inflammatory response in addition to local aortic damage. However, this reflects the clinical situation, where “target” tissue IRI provokes a proinflammatory and procoagulant reaction that will inevitably affect the microvasculature within the ischemic tissue as well as blood vessels, that, for instance in the case of vascular surgical procedures, themselves may be subject to a certain level of ischemic (or other) damage.

In ischemia/reperfusion injury (IRI), complement activation and the effectiveness of its inhibition in providing protection to various organs has been demonstrated in a multitude of models. Here we show that the glycosaminoglycan analog DXS attenuates IRI-induced aortic complement activation and damage and reduces apoptosis within the ischemic/reperfused segment. These results are in line with our own work and previous studies from others, where the use of glycosaminoglycan analogs such as heparin, reduced IRI through complement inhibition and endothelial association.

Whilst glycosaminoglycans, including heparin as well as cell-surface HSPG, have been reported to modulate MAPK signaling, complement itself influences MAPK activation. Sublytic amounts of C5b-9 activate the JNK and ERK pathways, inducing mitogenic signaling in a B-cell line and smooth muscle cell proliferation in aortas. Also, constitutive

![Representative images from immuno-histochemical staining for nuclear factor (NF) kappa B reveal reduced nuclear staining in native/normal and DXS aortas as compared to NaCl control aortas. Nuclear positivity appears as a brown staining (see arrow heads in inlay). Scale bar represents 50μm. Quantification of staining intensity using Image J software. The graph represents the total area of positivity (brown nuclei) for NF kappa B staining on aortic samples. Bars represent mean ± standard deviation.](http://doc.rero.ch)
ERK activation may exacerbate complement-mediated injury to glomerular epithelial cells. Inhibition of MAPK activation, for instance the stress-activated JNKS and p38-MAPKs, has been shown to reduce (complement-mediated) injury in various target organs and models. To date, however, it remains unknown, whether complement inhibition and accompanying modulation of MAPK signaling may be beneficial in reducing IRI-induced tissue injury. Our results indicate that complement inhibition by DXS is associated with reduced JNK and ERK1/2 activation. The latter effect by itself may provide one way by which DXS modulates the proinflammatory events associated with ischemia and reperfusion. The reduction in MAPK activation is accompanied by reduced tissue factor expression in the DXS treated aortas and correlated with reduced NF κB nuclear translocation, as we have already previously reported in an in vitro setting. Whether DXS also significantly affects the expression of intercellular adhesion molecules remains to be investigated. Influencing signaling pathways through modulation of protein kinases may prove an effective strategy to tackle a variety of diseases. However, there remains a degree of uncertainty as to whether inhibition or indeed activation of MAPK, proves beneficial or detrimental. In particular, effects may also be species- and model-dependent.

Binding of DXS to the damaged endothelium and aortic wall, visualized using fluorescein-labeled DXS, was observed following local administration. Binding was localized to ischemically damaged tissue and was not detected in non-ischemic aorta or other organs. DXS was also detected on the endothelium and within the aortic wall following pretreatment of the aorta with heparanase III, which, like other enzymes removes HSPG from the endothelial surface. This finding correlates with previous work, demonstrating increased binding of DXS to porcine endothelial cells following heparanase treatment.

The pharmacologically-induced HSPG release mimicked the HSPG loss detectable after 90 minutes ischemia, which was amplified following subsequent reperfusion. DXS largely prevented reperfusion-induced HSPG shedding. However, the increased staining intensity after DXS administration (Fig 3) may at least partly be due to cross-reaction of the anti-heparan sulfate antibody with endothelial-bound DXS. Cross-reactivity of this antibody with DXS was recently confirmed in a competitive inhibition experiment. Heparan sulfate, like medicinal heparin, enhances the activity of antithrombin, thereby contributing to the inactivation of thrombin and activated FXa, respectively. Heparanase treatment of endothelial cells reduces antithrombin binding, suggesting that this interaction is indeed mediated by heparan sulfate. Replacing the shed HSPG layer by a glycosaminoglycan analog, such as DXS, or indeed, preventing reperfusion-induced shedding, may help restore this essential property of “surface regulation” of the coagulation system.

In conclusion, we provide evidence that low molecular weight DXS substantially reduces endothelial damage following ischemia and reperfusion in an infrarenal aortic clamping model in rats. DXS provides cytoprotection through association with denudated endothelial and local complement inhibition, and modulates signaling through MAPK pathways in this setting. Local cytoprotection of damaged endothelium using substances such as DXS, which functionally restore the anti-inflammatory and anti-coagulant properties of the luminal glycosalumy, may provide a novel way to attenuate endothelial reperfusion-induced injury in the clinical setting.

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**AUTHOR CONTRIBUTIONS**

Conception and design: YB, RR
Analysis and interpretation: YB, TG, ZY, RR
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Writing the article: YB, RR
Critical revision of the article: YB, TG, KM, ZY, RR
Final approval of the article: YB, TG, KM, ZY, RR
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