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Chapter 7

Effect of chronic hyaluronidase infusion on kidney function and atherosclerosis progression in ApoE-deficient mice

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ABSTRACT

Background: The protective barrier between flowing blood and the vessel wall is formed by the endothelial glycocalyx. Damage to the glycocalyx causes increased permeability and loss of anti-atherogenic capacity of the vessel wall in experimental models. Effects of long-term glycocalyx damage in vivo remains to be established. Therefore, we evaluated the effect of chronic damage to glycocalyx through enzymatic degradation of hyaluronan, a major component, on renal barrier function and atherosclerosis progression.

Methods: Glycocalyx damage was inflicted by continuous infusion of active hyaluronidase (10 U/hr) for 4 weeks via an osmotic minipump in male ApoE-deficient mice (14 wks) on a Western type diet for 10 weeks (started at 8 wks of age). Control infusion consisted of heat-inactivated hyaluronidase. Systemic glycocalyx volume (Vg) was estimated by subtracting calculated plasma volume, estimated dilution of infused labeled erythrocytes and systemic hematocrit, from the dextran 40 kDa distribution volume. Plasma clearance of various sizes of dextran and ficoll as well as urine protein/creatinine ratio was determined. Plaque area and composition in the aortic arch were quantified to determine atherosclerosis progression.

Results: In the active hyaluronidase group, Vg was reduced from 21.6 ± 6.3 mL/kg body-weight (BW) (n = 6) at baseline to 12.5 ± 9.1 mL/kg BW (n = 15, p = 0.04), whereas inactivated hyaluronidase had no significant effect (18.3 ± 10.0 mL/kg BW, n = 14, ns). Chronic hyaluronidase infusion in the atherogenic ApoE-deficient resulted in impaired clearance of both low- and high molecular weight tracers which was accompanied by a doubled urine protein/creatinine ratio without apparent morphologic changes in glomerular and tubular histology. Total plaque area increased significantly from the onset of the Western-type diet, but no significant difference was observed between active and inactivated hyaluronidase treated mice. The latter may in part be due immune activation following infusion of both activate and inactivated hyaluronidase.

Conclusion: The profound glycocalyx disturbance following chronic hyaluronidase infusion in ApoE-deficient mice results in functional loss of vascular volume, accompanied by reduced systemic clearance of both small and large molecules and a relative increase in urinary protein excretion. These findings suggest that prevention of glycocalyx damage may serve as a potential target to protect vascular integrity and in particular its protein barrier properties.

Keywords: endothelial glycocalyx, hyaluronidase, proteinuria, atherosclerosis
INTRODUCTION

The protective barrier between flowing blood and the vessel wall is formed by the endothelial glycocalyx (1). Hyaluronic acid and negatively charged heparan sulphate proteoglycans are its major constituents. The volume of the glycocalyx depends on the balance between biosynthesis and degradation or shear-dependent shedding of its components (2). Historically, this layer was thought to be confined to a thickness of only several nanometers. More recently, intravital microscopy studies demonstrated intraluminally glycocalyx dimensions to reach up to 0.5-3 μm (3, 4).

Functional studies showed that disruption of the endothelial glycocalyx is accompanied by enhanced sensitivity of the vasculature towards atherogenic stimuli. These comprise increased vascular permeability as well as increased adhesion of leukocytes and thrombocytes to the vessel wall (5-7) and impaired NO release (8, 9). These observations are of particular interest since altered vascular permeability and attenuated NO bioavailability are amongst the earliest characteristics of atherogenesis (10). In large vessels, low-shear regions covered by a thin glycocalyx as opposed to high-shear regions have been found to be accompanied by significant swelling of the subendothelial matrix (11). However, in spite of these observations it has been proven difficult to show direct relevance of the endothelial glycocalyx as a protective layer in the atherogenic process in large vessels.

In addition, atherogenic stimuli are believed to attenuate the microcirculation and add to the vulnerability of peripheral organs and tissues. One such organ in particular might be the kidney, in which the endothelial glycocalyx might play an active role in glomerular barrier properties and anti-leukocyte adhesive properties of the peri-tubular endothelium. The glomerular filtration barrier, which consists of podocytes, glomerular basement membrane, fenestrated glomerular endothelial cells and a luminal endothelial glycocalyx, is perturbed upon specific enzymatic degradation of the glycocalyx which results in an increase in fractional clearance for albumin (12). This was confirmed in vitro using cultured glomerular endothelial cells, in which enzymatic removal of the glycocalyx more than doubled albumin flux through the endothelial lining (13).

We hypothesized that increased degradation of the endothelial glycocalyx early on in the development of atherosclerosis might accelerate disease progression. In the present study, in ApoE-deficient mice, on a Western-type high fat, high cholesterol diet for 10 weeks, received an osmotic mini-pump to intravenously infuse either active or inactive testicular hyaluronidase for the last 4 weeks. Systemic total vascular perfusion- and glycocalyx volumes, and plasma clearance rates of high and low molecular weight tracers were estimated to investigate systemic changes upon chronic damage to the endothelial glycocalyx. In addition,
renal barrier function and atherosclerosis progression in the aortic arch were evaluated to investigate local changes.

**METHODS**

**Mouse model and hyaluronidase infusion**

Male ApoE-deficient (ApoE<sup>−/−</sup>) mice (Charles River Laboratories, France) were fed a Western diet (semi-synthetic cholesterol-rich diet, containing 15% [w/w] fat and 0.25% [w/w] cholesterol), Diet W; Hope Farms, Woerden, the Netherlands) from the age of 14 weeks onwards. At 16 weeks an osmotic minipump (Alzet minipump 2004, DURECT Corporation) was implanted with a catheter (Alzet mouse jugular catheter #0007700) in the right jugular vein. Animals were anaesthetized using isoflurane and received temgesic for pain relieve. The minipump was filled with filtered active or heat inactivated hyaluronidase (bovine testis hyaluronidase, fraction IV-S, Sigma) dissolved in 0.9% NaCl, ensuring a constant dose of 10 U/hr intravenously for maximal 4 weeks. This dose has been found to reduce systemic glycocalyx volume without apparent morphologic changes in glomerular and tubular histology (data not shown). Measurements were performed at 8 weeks (baseline) (n = 6) and at 18 weeks, after 10 weeks of Western-type diet and the 4 weeks infusion of active hyaluronidase (n = 15) or inactive hyaluronidase (n = 14). All procedures were approved by the ethical committee and in accordance with national regulations on animal experiments. Hyaluronidase activity of active and inactivated hyaluronidase activity was determined using substrate gel electrophoresis (14).

**Estimation of systemic glycocalyx volume and dextran clearance**

The endothelial glycocalyx allows limited access to plasma macromolecules and erythrocytes, whereas smaller tracers can permeate into the glycocalyx (15). Systemic glycocalyx volume (V<sub>G</sub>) was estimated by tracer dilution techniques using labeled erythrocytes and dextran 40 kDa. We estimated V<sub>G</sub> by subtracting circulating plasma volume from the intravascular distribution volume of a glycocalyx permeable tracer, i.e. neutral dextran 40 kDa. The intravascular distribution volume of labeled erythrocytes was used to quantify circulating blood volume (16). For comparison, the distribution volume of dextran 500 kDa and ficoll 400 kDa was also determined. Glycocalyx barrier properties were estimated from the systemic clearance of dextran 500 kDa, ficoll 400 kDa compared with the clearance of dextran 40 kDa.

In summary, a catheter was inserted in the left jugular vein. To quantify circulating plasma volume, blood was labeled using 0.3 mg/ml of 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen). Dextran 40 kDa was used as a probe to estimate the intravascular volume including the glycocalyx compartment. 100 ul of suspension containing labeled erythrocyte and fluorescent labeled 10 mg/ml dextran 40 kDa (Texas Red labeled
dextran 40, Sigma), 5 mg/ml dextran 500 kDa (FITC labeled dextran 500, Sigma) and 50 mg/ml ficoll 400 kDa (TdB, Sweden), were infused. Blood samples were drawn in heparinized capillaries through tail bleeding before infusion as well as 2, 5, 10, 15, 20 and 30 minutes after infusion. Capillaries were centrifuged, hematocrit was determined, and plasma collected and stored at -20°C until fluorescence analysis. The circulating fraction of labeled erythrocytes was measured using flowcytometry (FACSCalibur; Becton Dickinson) to estimate the total circulating erythrocyte volume (V_{ERY}). Circulating plasma volume was calculated from V_{ERY} and large vessel hematocrit (Ht) by the following formula: ([1 – Ht] x V_{ERY})/Ht. In each sample, fluorescence was measured at 590 nm for Dextran 40, at 535 nm for Dextran 500 and 420 nm for ficoll 400 with a spectrophotometer (VICTOR; Perkin-Elmer). Dextran concentrations calculated in reference to defined dilutions of the infused tracer mix in plasma from donor mice. Concentration–time curves of all tracers were fitted with a monoexponential function. Initial distribution volume of each tracer was determined from the extrapolated dilution at the start of tracer injection, and clearance was defined as the percentage decrease in tracer concentration at the final sample point (t = 30 minutes) compared with the extrapolated concentration at the start of tracer injection (t = 0 minutes).

**Biochemical parameters**

Lipid distribution over plasma lipoproteins was determined by fast performance liquid chromatography in 80 μL pooled plasma. IL-6 was measured in duplo using a commercially available assay (Mouse IL-6 BD optEIA, Becton Dickinson). A value of 1000 was assigned to levels exceeding 1000 pg/mL for further calculations. Glucose was measured before isoflurane anaesthesia using a glucometer (Accu-Chek, Roche). Serum creatinin was measured by Jaffé kinetic colorimetric test (Roche Diagnostics) on Modular P800 (Roche Diagnostics). Urinary creatinin and protein content was determined according to the Jaffé method on the P800 and by immunoturbidimetric assay, respectively (Roche Diagnostics).

**Hyaluronidase antibody detection**

In a subset of mice, we determined whether antibodies against bovine hyaluronidase had been formed using the following assay. First 96 wells plates (Nunc Maxisorb) were coated for 1 hr at 37°C with bovine hyaluronidase (Sigma) in a concentration of 100 U/ml. Blockage was performed using 3% goat milk in PBS for 1 hr at 37°C. After blockage mice serum samples (diluted 10x, 100x, 1000x, 10 000x, 100 000x and 1 000 000x in PBS) were incubated for 1 hr at 37°C. Serum of naïve mice was used as negative control. Mouse-anti-rat/bovine hyaluronidase antibody (Mouse Anti-Hyaluronidase PH20 Monoclonal Antibody, Clone 1D6, Abcam, concentration 10 μg/mL) was used as a positive control. After incubation mouse-anti hyaluronidase antibodies were detected using GAMPO (Goat-anti-Mouse Peroxidase, DAKO, 1:2000). Substrate (OPD (Sigma)/H2O2 in PO4- citrate buffer) was added for 20 minutes. The
reaction was terminated using 2 M H$_2$SO$_4$. Extinction was measured at 490 nm. Between each step plates were washed 5 times using PBS/0.05% Tween.

**Tissue processing**

Mice were perfused through the apex of the heart for 10 minutes with phosphate-buffered saline (PBS) containing sodium nitroprusside (Sigma) and for 10 min with 1% phosphate-buffered paraformaldehyde, pH 7.4. The heart, aortic arch and its side branches as well as kidneys, liver, spleen and lung tissue were removed and fixed overnight in 1% phosphate-buffered paraformaldehyde. Subsequently, tissue was processed and embedded in paraffin. Forty serial sections of the aortic arch, 4 μm each, were cut and used for analysis.

**Histochemistry**

Sections of kidney, liver, spleen and lung tissue were stained with hematoxylin and eosin (HE) and evaluated for morphologic abnormalities. Four sections of the aortic root, 40μm apart, were stained with HE and were used for determining plaque area. Tissue sections were stained for collagen content (Sirius red) and macrophage content (Mac-3, 1:30, BD-Pharmingen 553322). All morphometric parameters were determined using a microscope coupled to a computerized morphometry system (Leica, Rijswijk, the Netherlands). Lesion area was determined as an average of four sections per mouse. The number of early lesions (fatty streaks containing only foam cells) and advanced lesions (showing foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis) was counted. The collagen content was expressed as positive area relative to the total plaque area, with special attention for the innominate artery (17).

**Statistical analysis**

Results are presented as mean ± SD. Data were analyzed using the unpaired, two-sided Student’s t test. Plaque area is presented as median [interquartile range] and analyzed using the Mann Whitney U test. A p-value of < 0.05 was considered statistically significant.

**RESULTS**

**Chronic infusion of hyaluronidase and diet**

ApoE$^{-/-}$ mice tolerated chronic infusion with hyaluronidase well. Zymography revealed abundant hyaluronidase activity in the activate hyaluronidase solution and none in the heat inactivated solution for infusion. Western type diet gave rise to high cholesterol levels, with a total cholesterol level of 4.69 mmol/L (pooled sample) at time of pump implantation (week 14). After 4 weeks of additional combined diet and infusion of hyaluronidase, total cholesterol further increased to 6.7 ± 1.9 mmol/L in the active group and 7.2 ± 1.7 mmol/L.
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in the inactive group. Blood glucose levels were 5.8 ± 1.0 mmol/L at baseline and did not change significantly (Active group: 7.8 ± 2.3 mmol/L (p = 0.07), inactive group: 6.9 ± mmol/L (p=0.50).

**Systemic glycocalyx volume**

At the start of the experiment, baseline ApoE -/- mice weighted 25.5 ± 0.8 g. Their average plasma volume was 0.85 ± 0.06 mL (n=6), with a dextran (40kDa) distribution volume of 1.4 ± 0.18 mL. This resulted in a baseline Vg of 21.6 ± 6.3 mL/kg bodyweight. After 10 weeks of Western type diet and 4 weeks of continuous infusion with 10 U/hr hyaluronidase, mice body weight increased to 28.0 ± 2.3 g (excluding minipump; p = 0.02 compared to baseline). Average plasma volume increased to 1.03 ± 0.18 (n = 15, p = 0.03), with an unchanged dextran distribution volume (1.39 ± 0.28 mL; p = 0.93). The resulting average Vg 12.5 ± 9.1 mL/kg (p = 0.04) was 42% lower than baseline levels (Figure 1). Following infusion of inactivated hyaluronidase, weight increased to 28.9 ± 1.9 g (excluding minipump; p = 0.001 compared to baseline). Average plasma volume increased to 1.27 ± 0.31 mL (n = 14, p = 0.005) with a significantly reduced hematocrit of 0.41 ± 0.03 (baseline Hct 0.45 ± 0.02, p = 0.01), whereas dextran volume tended to increase as well to 1.80 ± 0.49 mL (p = 0.08). Consequently, Vg was 18.3 ± 10.0 mL/kg (p = 0.47, compared to baseline), similar to baseline values (Figure 1).

![Graph showing plasma, red blood cell and glycocalyx volume in ApoE-/- mice treated with active or inactivated hyaluronidase](image)

**Figure 1.** Plasm, red blood cell and glycocalyx volume in ApoE-/- mice treated with active or inactivated hyaluronidase.

Chronic infusion of hyaluronidase in ApoE-/- mice reduced Vglycocalyx and was accompanied by a loss of total vascular volume (Vtotal, defined as dextran 40 distribution volume) (p < 0.05 vs. ApoE-/- without infusion).

**Kidney function**

At baseline protein/creatinine ratio was 0.21 g/mmol (pooled sample). This increased to 0.39 g/mmol in mice treated with active hyaluronidase. In the inactive group, the ratio was comparable to baseline values (0.23 g/mmol). Serum creatinine was 19 μmol/L at baseline as well as in the inactive group and 23 μmol/L in the active group. Figure 2 shows the average clearance (abs - [1/t]) of dextran 40, dextran 500 and ficoll 400. Clearance of dextran 40 and 500 was decreased in the active group compared to the inactivated group (both p = 0.05).
Kidney morphology appeared normal, including normal appearance of glomerular tissue and tubular epithelial cells and no apparent loss of nephrons or sequestration of immune complexes.

Inflammatory response
Infusion of hyaluronidase, both active as well as inactivated, elicited an immune response. Antibodies titers against bovine hyaluronidase between 0.32 and 0.74 g/L were found in both groups. These levels were 230 to 530 fold higher than those in naïve ApoE−/− mice. Levels of IL-6, an inflammatory cytokine, increased from 146 ± 65 pg/mL at baseline to 413 ± 246 pg/mL in the active group (p = 0.02) and 678 ± 320 pg/mL in the inactive group (p < 0.001), respectively. Inspection of tissue morphology revealed inflammatory activity in the liver, i.e. mild polymorphonuclear cell infiltration and spindle cell proliferation as well as mild steatosis, of mice from the active as well as the inactive group. In some cases, inflammatory activity was also observed in the spleen. Lung tissue did not show abnormalities.

Atherosclerosis progression
ApoE−/− mice before start of Western type diet (baseline, 8 weeks of age) already contained small fatty streak lesions within the aortic arch with a median total plaque area of 0.7 [0.3 - 1.1] mm². After the full period of Western-type diet and enzyme infusion significant (p < 0.05) advanced lesions with intimal thickening, cholesterol accumulation and necrotic cores were observed in both the active- and inactive hyaluronidase group with a total plaque area of 20.2 [15.0 – 26.1] mm² and 17.7 [12.1 -26.2] mm² respectively. There was no significant difference between the active and the inactive group (p = 0.96). Median collagen content was 31.5 ± 11.7% in the active group and 31.5 ± 17.5% in the inactive group (ns). Disruption of the intima border/lesion border was present in 7 out of 8 mice in the active group vs. 0 out of 8 in the inactive group.

Figure 2. Clearance rates of 40 & 500 kDa dextran tracers in ApoE−/− mice upon hyaluronidase treatment were reduced, with little effect on 400 kDa ficoll clearance (* p < 0.05 vs. hyaluronidase inactive).
DISCUSSION

In the present study, we show for the first time that chronic hyaluronidase infusion via an osmotic minipump results in a persistent reduction in systemic glycocalyx volume approximating 40%. This change in glycocalyx volume was accompanied by a doubling in urinary protein/creatinine ratio and reduced dextran clearance, whereas renal morphology remained intact. In contrast, in both the active as well as the inactive group advanced atherosclerotic lesions developed with a comparable plaque area. However, antibodies against bovine hyaluronidase, high IL-6 levels as well as signs of inflammatory activity in liver and spleen were present in both the active and inactivated hyaluronidase group. This inflammatory response may have blurred the interpretation of our results. Our study warrants another approach to establish the role of the glycocalyx in atherosclerosis progression and further investigation to address the role of the glycocalyx in the kidney.

Systemic glycocalyx volume
To estimate glycocalyx volume we subtracted plasma volume, determined using labeled erythrocytes, from the distribution volume of the glycocalyx penetrating tracer dextran 40. In previous experiments, an acute bolus of hyaluronidase profoundly reduced glycocalyx thickness (18, unpublished data). In the present study, we show that chronic hyaluronidase infusion induces sustained glycocalyx damage. Although antibodies were formed against hyaluronidase, this most likely did not completely block its activity as infusion of active and not inactivated hyaluronidase reduced systemic glycocalyx volume compared to baseline.

In detail, dextran volume was significantly smaller in the active hyaluronidase group compared to the inactive group (1.39 ± 0.28 mL vs. 1.80 ± 0.49 mL, p = 0.01), whereas plasma volume expanded modestly in both groups. This was accompanied by a small drop in hematocrit (baseline hematocrit: 0.45 ± 0.02; active group: 0.43 ± 0.03 (p = 0.08); inactive group: 0.41 ± 0.03 (p = 0.01). The mechanism behind this plasma expansion is unclear, but could be caused by an infusion-related increase in plasma osmolality.

Previously, the use of labeled erythrocytes in afore-mentioned equation has been suggested to potentially underestimate plasma volume thus contributing to an overestimation of glycocalyx volume. Therefore, we verified the data using the distribution volume of dextran 500 kDa (19). Indeed, this resulted in a smaller systemic glycocalyx volume, but the effect of active hyaluronidase infusion was still clearly present (6.7 ± 3.0 mL/kg at baseline, 1.8 ± 4.7 mL/kg after infusion). More importantly, the ratio between plasma volume estimated using dextran 500 versus labeled erythrocytes was comparable between baseline and post-infusion measurements (Baseline: 1.4; active hyaluronidase: 1.3; inactive hyaluronidase: 1.4, ns). This indicates that our method is not affected by changes in kidney function.
Renal barrier function

We observed an increased protein/creatinine ration as well as a decreased dextran clearance in the active hyaluronidase group compared to the inactive group. In general, there are four mechanisms of excessive urine protein excretion, i.e. (i) altered glomerular permeability resulting in increased filtration of normal plasma proteins such as albumin, (ii) inadequate tubular reabsorption of small amounts of normally filtered proteins, (iii) glomerular filtration of circulating abnormal amounts of small proteins that exceed the reabsorption capacity of the tubules and finally, (iv) increased secretion of tissue proteins associated with inflammatory or neoplastic conditions (20). The first, so-called glomerular proteinuria, due to increased filtration of macromolecules across the glomerular capillary wall, is the most likely mechanism in this case. Mechanisms of glomerular filtration have been a matter of controversy for several decades. However, proteinuria may occur regardless of which layer of the glomerular wall (podocyte, glomerular basement membrane, endothelial cell or endothelial glycocalyx) is damaged (21, 22).

The selective increase in protein/creatinine ratio following active hyaluronidase implies a direct role for the glycocalyx. In accordance, in vitro models have confirmed that enzymatic degradation of the glycocalyx increases albumin flux (13). Interestingly, systemic glycocalyx perturbation in diabetic patients was most severe in those suffering from microalbuminuria (23). We cannot exclude that hyaluronidase also affected the glomerular basement membrane, but the intravenous route of administration limits direct exposition of the basement membrane to the enzyme. Besides that, the decrease in dextran clearance may indicate a reduction in glomerular filtration rate. Loss of glycocalyx may lead to capillary closure (24) and reduce the number of functional nephrons.

Atherosclerosis progression

In the present study we did not find a difference in atherosclerotic lesion area or collagen content following active versus inactivated hyaluronidase infusion. In view of existing experimental evidence, this was not in line with expectation. Although most glycocalyx research has been performed in the microvasculature, studies in the macrovasculature showed that a small glycocalyx thickness is accompanied by significant swelling of the subendothelial matrix (11). Similarly, extravasation of low density lipoprotein (LDL) cholesterol particles into the subendothelial space can be limited by the glycocalyx (25).

A complicating factor in evaluating the difference in atherosclerosis progression pertains to the fact that all animals, whether receiving active or inactivated hyaluronidase, were characterized by a profound inflammatory response. The increase in antibody titers against hyaluronidase was accompanied by a systemic inflammatory response, illustrated by a 2.8 to 4.6 fold increase in IL-6 levels. Since inflammation is right at the core of accelerated athero-
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genesis (26), we cannot exclude that the inflammatory impetus may have overruled potential impact of glycocalyx perturbation on atherosclerosis progression. Moreover, it is difficult to estimate the exact impact of this inflammatory activity. On the one hand, LPS-induced inflammation aggravates atherosclerosis in ApoE−/− mice (27). However, reports on the effect of IL-6 on atherosclerosis in ApoE−/− are mixed (28, 29). Therefore, this particular question needs further validation in a model using other mechanisms to inflict glycocalyx perturbation. Use of mice deficient for hyaluronan synthase in endothelial cells might circumvent these problems.

Study limitations
Our model appeared to be unsuited to selectively examine the effect of glycocalyx damage on atherosclerosis progression, as it was accompanied by an inflammatory response. The immune response elicited by both active and inactive bovine hyaluronidase as well as the potential formation of small, pro-inflammatory hyaluronan fragments by hyaluronidase activity limit the use of hyaluronidase for this purpose (30). Although inflammation might also affect kidney function, we expect the inflammatory activity in both groups to lead to underestimation of the difference between the active and inactive group at most.

CONCLUSION

Chronic infusion of hyaluronidase reduces glycocalyx volume in ApoE−/− mice. This accompanied by increased urine protein/creatinine ratio and reduced dextran clearance, supporting the potential role of the glycocalyx in the kidney. Another study design is warranted to evaluate the specific effects of loss of endothelial glycocalyx on atherogenesis as the effects of glycocalyx damage and inflammatory activity are entangled.

DISCLOSURES

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