Chronic Hyperglycemia Attenuates Coronary Collateral Development and Impairs Proliferative Properties of Myocardial Interstitial Fluid by Production of Angiostatin

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Background—Development of coronary collateral vessels is impaired in patients with diabetes mellitus. We tested the hypothesis that hyperglycemia alone attenuates collateral development and abolishes proliferative properties of myocardial interstitial fluid (MIF) by enhancing expression of matrix metalloproteinases (MMP) and angiostatin.

Methods and Results—Chronically instrumented dogs were randomly assigned to receive an infusion of normal saline (control; n=9) or 70% dextrose in water to increase blood glucose to 350 to 400 mg/dL for 8 h/d (hyperglycemia; n=7) in the presence or absence (sham; n=9) of brief (2 minutes), repetitive coronary artery occlusions (1/h; 8/d for 21 days). Collateral perfusion increased to 41±11% and 49±6% of normal zone flow in control dogs on days 14 and 21 (P<0.05) but remained unchanged over 21 days in hyperglycemic and sham dogs (12±3% and 13±3%, respectively). A progressive reduction of the postocclusive peak reactive hyperemic response was also observed in control dogs (16±1 to 10±1 Hz·10^2 on days 1 and 21, respectively) but not in hyperglycemic (17±2 to 20±2) or sham (17±2 to 16±1) dogs. Endothelial cell tube formation was produced by MIF obtained from control dogs but not hyperglycemic or sham dogs. Coincubation of MIF from hyperglycemic dogs with an angiostatin antibody restored endothelial cell tube formation. MMP-9 activity and expression of angiostatin were increased in dogs receiving exogenous glucose compared with controls.

Conclusions—Chronic hyperglycemia abolishes development of coronary collateral vessels by increasing MMP-9 activity and angiostatin expression in dogs. (Circulation. 2004;109:2343-2348.)

Key Words: angiogenesis ■ collateral circulation ■ diabetes mellitus ■ glucose ■ metalloproteinases
the guiding principles for research involving animals and human beings of the American Physiological Society and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**General Preparation**

Dogs were instrumented as previously described. Under isoflurane anesthesia, a left thoracotomy was performed in the fifth intercostal space, and a segment of the left anterior descending coronary artery (LAD) distal to the first diagonal branch was isolated. A Doppler flow transducer was placed around the LAD for measurement of blood flow velocity. A hydraulic vascular occluder was placed immediately distal to the flow transducer for production of repetitive coronary artery occlusion and reperfusion. Peak reactive hyperemic response was measured as the peak Doppler frequency shift occurring immediately after LAD occlusion and reperfusion. Heparin-filled catheters were secured in the descending thoracic aorta and the right atrium for measurement of aortic blood pressure and fluid administration, respectively. A catheter was placed in the left atrial appendage for administration of radioactive microspheres. A multiport catheter was implanted in the midmyocardium of the LAD perfusion territory for sampling of myocardial interstitial fluid (MIF). Each dog was permitted to recover 7 to 10 days and was then trained to stand in a sling during daily hemodynamic monitoring.

**Experimental Protocol**

Coronary collateral development was induced with brief (2 minutes), repetitive (1/h; 8/d for 21 days) LAD occlusions. Systemic and coronary hemodynamics were monitored daily and recorded on a polygraph. Radioactive microspheres were administered during the first 2 minutes of LAD occlusion on experimental days 1, 7, 14, and 21. Dogs were randomly assigned to receive an infusion of normal saline, a left thoracotomy was performed in the fifth intercostal space, and a segment of the left anterior descending coronary artery (LAD) distal to the first diagonal branch was isolated. A Doppler flow transducer was placed around the LAD for measurement of blood flow velocity. A hydraulic vascular occluder was placed immediately distal to the flow transducer for production of repetitive coronary artery occlusion and reperfusion. Peak reactive hyperemic response was measured as the peak Doppler frequency shift occurring immediately after LAD occlusion and reperfusion. Heparin-filled catheters were secured in the descending thoracic aorta and the right atrium for measurement of aortic blood pressure and fluid administration, respectively. A catheter was placed in the left atrial appendage for administration of radioactive microspheres. A multiport catheter was implanted in the midmyocardium of the LAD perfusion territory for sampling of myocardial interstitial fluid (MIF). Each dog was permitted to recover 7 to 10 days and was then trained to stand in a sling during daily hemodynamic monitoring.

Radioactive microspheres (15–2 μm in diameter) labeled with 141Ce, 109Ru, 51Cr, or 95Nb were used to measure regional myocardial blood flow, as previously described.

**Angiostatin Expression**

Western blotting was used to assess the expression of angiostatin in MIF. Twenty microliters of interstitial fluid was loaded for electrophoresis. After electrophoretic transfer to a nylon membrane, the blot was blocked with 3% nonfat dry milk. The antibody was then incubated in 3% nonfat dry milk for 1 hour at room temperature, followed by several washes and incubation with a secondary antibody. Detection of the bands was achieved with nonradioactive chemiluminescence. A 1-μg standard of the plasminogen protein was run for comparison with the proteins in the interstitial fluid sample. Signals were scanned with a Fluorimager, and the density was analyzed with the use of ImageQuant. All samples were studied in triplicate.

**Cell Cultures**

Human aortic endothelial cells (EC) were grown to confluence in Ham/F12 media (GIBCO) and supplemented with 15% fetal bovine serum (FBS), penicillin (500 U/mL), streptomycin (50 μg/mL), heparin (100 μg/mL) (all from GIBCO), and EC growth factor (20 μg/mL; Boehringer Mannheim) in the presence of 95% air/5% CO₂ at 37°C. Rat pulmonary arterial vascular smooth muscle cells (VSMC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, penicillin, streptomycin, L-glutamine, and sodium pyruvate.

**Cell Proliferation**

The mitogenic activity of MIF was assessed at days 1, 7, 14, and 21 by determining the proliferative response of growth-arrested EC and VSMC grown in culture. Ten thousand cells per well were seeded in DMEM containing 5% FBS overnight. The cells were growth arrested for 72 hours in 0.5% FBS for EC or 0.1% FBS for VSMC in DMEM. The cells were then incubated with a 10% dilution of MIF (200 μL MIF diluted in 1800 μL of 0.5% or 0.1% DMEM, respectively). Other cultures were treated with 0.5% or 0.1% FBS as negative controls and 10% FBS as positive controls for the same time interval. After 72 hours, the number of cells was determined with a hemacytometer.

**In Vitro Angiogenesis Assay**

Endotoxin- and plasminogen-free bovine fibrinogen (5 mg/mL; Calbiochem) was dissolved in serum-free medium and filtered through 0.2-μm filters (Millipore, Millipore). Fibrin matrices were prepared by polymerizing the fibrinogen solution with the use of a low concentration of α-thrombin (2.5 U/mL; Sigma). After polymerization, gels were soaked in culture medium containing 10% FBS for 2 hours at 37°C to inactivate the thrombin. EC were seeded (40 000 cells per well) on the surface of the 3-dimensional matrix in 24-well plates in Ham/F12 media and cultured for periods up to 48 hours in the presence or absence of MIF from sham, hyperglycemic, and control dogs. A polyclonal angiostatin antibody (4 μg/mL; Pharmingen) was incubated with the interstitial fluid of sham and hyperglycemic dogs. Angiogenic properties were determined by calculating total area of tubes formed after superimposing the image on a predetermined grid and assessing the number of squares (0.25 μm²) containing intersecting tubes.

**Gelatin Zymography**

Gelatinolytic activity of MMP-9 was analyzed by using gelatin zymography. Twenty-five microliters of MIF in 25 μL of zymogram sample buffer (BioRAD) was applied to a 10% polyacrylamide gel copolymerized with 1 mg/mL gelatin (BioRAD). After electrophoresis (constant current of 120 mV), the gel was washed for 1 hour in 2.5% Triton X-100 (Renaturation Buffer BioRAD) at room temperature and incubated overnight in 50 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl, 5 mmol/L CaCl₂, and 0.02% Brij-35 (Development Buffer BioRAD). Gels were stained with 0.5% Coomassie blue in 30% methanol and 10% glacial acetic acid and destained in the same solution lacking Coomassie blue. One nanogram of recombinant MMP-9 was used as a standard. The zymograms were visualized with an Alpha Imager and analyzed densitometrically with Scion Image.

**Statistical Analysis**

Statistical analyses of data were performed with repeated-measures ANOVA followed by Student-Newman-Keuls test. Data are expressed as mean±SEM and were considered statistically significant at P<0.05.

**Results**

**Hemodynamics**

There were no differences in heart rate (HR), mean arterial pressure (MAP), rate-pressure product (RPP), and peak reactive hyperemia (PRH) between groups under baseline conditions (Table 1). No temporal changes in HR, MAP, and RPP were observed in control and sham dogs. In contrast, HR and RPP increased in hyperglycemic dogs on day 21 compared with day 1. A progressive reduction in PRH was observed in control dogs over the time course of repetitive ischemic stimuli (16±2 to 10±1 Hz · 10⁷ on days 1 and 21, respec-
TABLE 1. Hemodynamics

<table>
<thead>
<tr>
<th>Experimental Day</th>
<th>1</th>
<th>7</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>99±9</td>
<td>84±11</td>
<td>85±14</td>
<td>94±5</td>
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<tr>
<td>Hyperglycemia</td>
<td>101±5</td>
<td>113±12</td>
<td>130±11†</td>
<td>157±18†</td>
</tr>
<tr>
<td>Sham</td>
<td>90±7</td>
<td>98±11</td>
<td>94±6</td>
<td>95±8</td>
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<tr>
<td>MAP, mm Hg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>98±4</td>
<td>94±5</td>
<td>98±4</td>
<td>96±6</td>
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<tr>
<td>Hyperglycemia</td>
<td>107±6</td>
<td>98±7</td>
<td>101±6</td>
<td>103±7</td>
</tr>
<tr>
<td>Sham</td>
<td>110±4</td>
<td>114±3</td>
<td>111±3</td>
<td>105±4</td>
</tr>
<tr>
<td>RPP, mm Hg·bpm·10³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.9±1.2</td>
<td>10.3±1.1</td>
<td>11.0±1.9</td>
<td>11.8±1.0</td>
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<tr>
<td>Hyperglycemia</td>
<td>13.5±0.9</td>
<td>14.4±0.2</td>
<td>17.4±0.1</td>
<td>22.6±0.4†</td>
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<tr>
<td>Sham</td>
<td>11.5±1.6</td>
<td>11.2±1.1</td>
<td>10.6±0.5</td>
<td>10.4±1.3</td>
</tr>
</tbody>
</table>

Data are mean±SEM (control group, n=9; hyperglycemia group, n=7; sham group, n=9). *Significantly (P<0.05) different from day 1. †Significantly (P<0.05) different from respective value in control dogs.

TABLE 2. Coronary Collateral Perfusion to Ischemic Region

<table>
<thead>
<tr>
<th>Experimental Day</th>
<th>1</th>
<th>7</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subepicardium</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.16±0.03</td>
<td>0.35±0.09</td>
<td>0.45±0.09*</td>
<td>0.68±0.13*</td>
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<td>Hyperglycemia</td>
<td>0.27±0.07</td>
<td>0.33±0.08</td>
<td>0.49±0.13</td>
<td>0.72±0.30</td>
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<tr>
<td>Sham</td>
<td>0.28±0.08</td>
<td>0.16±0.05</td>
<td>0.16±0.04</td>
<td>0.18±0.03†</td>
</tr>
<tr>
<td>Midmyocardium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.10±0.01</td>
<td>0.40±0.16</td>
<td>0.61±0.23*</td>
<td>0.80±0.16*</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>0.08±0.03</td>
<td>0.14±0.06</td>
<td>0.16±0.04</td>
<td>0.33±0.12†</td>
</tr>
<tr>
<td>Sham</td>
<td>0.07±0.01</td>
<td>0.09±0.03</td>
<td>0.09±0.02</td>
<td>0.09±0.02†</td>
</tr>
<tr>
<td>Subendocardium</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.15±0.03</td>
<td>0.40±0.14</td>
<td>0.62±0.21*</td>
<td>0.77±0.16*</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>0.08±0.03</td>
<td>0.09±0.02†</td>
<td>0.09±0.03†</td>
<td>0.17±0.08†</td>
</tr>
<tr>
<td>Sham</td>
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<td>0.04±0.02†</td>
<td>0.05±0.02†</td>
<td>0.05±0.01†</td>
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<tr>
<td>Transmural</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.14±0.02</td>
<td>0.38±0.13</td>
<td>0.56±0.17*</td>
<td>0.75±0.14*</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>0.14±0.03</td>
<td>0.19±0.05</td>
<td>0.25±0.06</td>
<td>0.41±0.17</td>
</tr>
<tr>
<td>Sham</td>
<td>0.12±0.03</td>
<td>0.09±0.03</td>
<td>0.10±0.02†</td>
<td>0.11±0.02†</td>
</tr>
</tbody>
</table>

Data are mean±SEM, expressed in milliliters per minute per gram. *Significantly (P<0.05) different from day 1. †Significantly (P<0.05) different from respective value in control dogs.

There were no differences in collateral perfusion between groups on day 1 (Table 2). Increases in collateral perfusion were observed in control dogs (day 1=0.14±0.02; day 14=0.56±0.17; day 21=0.75±0.14 mL·min⁻¹·g⁻¹), but collateral perfusion was unchanged over time in hyperglycemic and sham dogs. Increases in coronary collateral blood flow (expressed as a percentage of normal zone flow) also occurred in control dogs (41±11% and 49±6%) compared with hyperglycemic dogs (12±4% and 12±3%) and sham dogs (9±3% and 13±3%) on days 14 and 21, respectively (Figure 1). Normal zone transmural perfusion was unchanged over time in control (1.29±0.09 and 1.54±0.18 mL·min⁻¹·g⁻¹ on days 1 and 21, respectively) and sham (1.12±0.09 and 1.07±0.18 mL·min⁻¹·g⁻¹) dogs. In contrast, a significant increase in normal zone perfusion occurred in hyperglycemic dogs on days 14 (2.53±0.28 mL·min⁻¹·g⁻¹) and 21 (3.35±0.59 mL·min⁻¹·g⁻¹) compared with day 1 (1.97±0.21 mL·min⁻¹·g⁻¹).

Figure 1. Coronary collateral blood flow to the ischemic region expressed as a percentage of normal zone flow. Collateral perfusion is increased over time in control dogs but not sham (no ischemia) or hyperglycemia-treated dogs. *Significantly (P<0.05) different from baseline; †significantly (P<0.05) different from the respective value in control dogs.
Proliferation of EC and VSMC
Comparisons of proliferative responses to MIF obtained after the initiation of repetitive ischemic stimuli are summarized in Figure 2. The mitogenic effect of MIF on VSMC was pronounced in control dogs. In contrast, interstitial fluid obtained from sham and hyperglycemia-treated dogs did not induce proliferation of VSMC. EC proliferation in response to MIF exposure followed a pattern similar to that observed for VSMC (Figure 3). MIF sampled from control dogs increased proliferation on days 7, 14, and 21 compared with growth-arrested (0.5% FBS) negative control experiments or MIF from sham and hyperglycemic dogs. Proliferation of EC did not occur in response to MIF obtained from sham and hyperglycemic dogs.

In Vitro Angiogenesis
EC tube formation in response to MIF obtained from control dogs was greater than negative controls throughout the experiment (Figure 4). Increases in EC tube formation were similar to levels observed in positive (50 ng of recombinant VEGF) control experiments. In contrast, MIF obtained from sham and hyperglycemic dogs did not produce EC tube formation. Stimulation of in vitro angiogenesis by MIF from hyperglycemic but not sham dogs was enhanced by treatment with angiostatin antibody.

MMP-9 Activity and Expression of Angiostatin
MMP-9 activity was upregulated in hyperglycemic compared with control dogs at early and late time points after the onset of repetitive ischemic stimuli (Figure 5). Western blot analysis of MIF of hyperglycemic and control dogs demonstrated that protein concentrations of angiostatin were increased after 1 week of brief, repetitive coronary artery occlusions (Figure 6). Angiostatin concentrations in hyperglycemic dogs were significantly greater than controls.

Discussion
The results of the present investigation demonstrate that chronic hyperglycemia exerts a profound negative impact on the development of the coronary collateral circulation in vivo. These data are supported by observations that the proliferation of EC and VSMC in vitro is absent after incubation with MIF obtained from hyperglycemic dogs. The results further indicate that the production of proteins known to inhibit angiogenesis may represent an important mechanism for the observed impairment of collateral development during hyperglycemia. Angiostatin concentrations were elevated in MIF collected from hyperglycemic dogs compared with their
euglycemic controls. Furthermore, the activity of MMP-9, a gelatinase responsible for formation of angiostatin from plasminogen, was increased in MIF in response to chronic hyperglycemia. These data suggest that production of angiostatin mediated by enhanced MMP-9 activity contributes to the attenuation of coronary collateral development in response to repetitive ischemic stimuli during chronic hyperglycemia.

Impairment of Coronary Collateral Development During Hyperglycemia

The present results confirm the findings of previous studies indicating that brief repetitive coronary occlusions under euglycemic conditions result in the development of a robust coronary collateral circulation over 21 days in dogs. In contrast, no increases in perfusion to ischemic myocardium occurred in the chronic presence of blood glucose concentrations >350 mg/dL, indicating that collateralization does not occur under these conditions. Angiographic studies conducted in patients with DM have established convincingly that these patients demonstrate impaired coronary collateral development. Thus, the present results suggest that impairment of collateral formation during persistent hyperglycemia may represent a mechanism for the well-known increased morbidity and mortality of coexisting DM and CAD.

Cell proliferation and migration are important hallmarks of angiogenesis. Our results confirm that MIF collected from euglycemic dogs undergoing repetitive ischemic stimuli causes dramatic proliferation of EC and VSMC in vitro. In addition, this fluid stimulates EC to form tubes in culture. Therefore, MIF contains growth factors that are critical to the angiogenic process. In contrast to these previous findings, MIF collected from chronically hyperglycemic dogs undergoing repetitive coronary occlusions did not stimulate either cell proliferation or tube formation. These present data are consistent with the results of other studies that have identified reductions in cell proliferation, increases in cell cycle duration, and enhanced apoptosis of EC in response to high concentrations of glucose.

Angiostatin Is Increased During Chronic Hyperglycemia

Angiogenesis relies on the enhanced production and release of growth factors (eg, vascular endothelial growth factor [VEGF]) and the reduced formation of growth inhibitors (eg, endostatin, angiostatin). We focused on the role of angiostatin in the present investigation because we have previously shown that abolition of nitric oxide production is associated with enhanced angiostatin expression and impaired collateral development. Considerable evidence also demonstrates that nitric oxide availability is substantially reduced during hyperglycemia. The present results indicated that MIF collected from chronically hyperglycemic dogs undergoing repetitive coronary occlusions contained greater concentrations of angiostatin compared with euglycemic controls. Furthermore, addition of an angiostatin antibody to MIF from hyperglycemia-treated dogs restored EC tube formation to levels similar to those observed with MIF collected from euglycemic dogs. These results provide support for a critical role of angiostatin in impaired angiogenesis during chronic hyperglycemia.

The precise mechanism(s) responsible for inhibition of coronary collateral development remains unclear. It is well established that angiostatin prevents EC proliferation and tumorigenesis, but few reports of the role of angiostatin in other pathological settings exist at present. Our present and previous findings support the contention that angiostatin concentrations may be adversely modulated and coronary collateralization may be impaired in various disease states, particularly those in which nitric oxide signaling is impaired. Angiostatin has been shown to be generated by macrophages, and these cells have important roles in wound healing,
angiogenesis, and chronic inflammation. Coronary collateral development is dependent to some degree on vasodilation, and angiostatin has been shown to impair both VEGF- and acetylcholine-induced vasodilation. However, the present results using an EC tube formation assay indicate that angiostatin contained in MIF exerts direct antiangiogenic actions in vitro independent of vasodilation or increases in normal zone coronary blood flow produced by chronic hyperglycemia.

MMP-9 Activity and Chronic Hyperglycemia
Plasminogen is cleaved by MMP-2, -7, or -9 to produce angiostatin. The production of angiostatin has been previously shown to be related to increased activity of MMP-2 and MMP-9 in a similar canine model of coronary collateral formation. The present results indicate that MMP-9 activity is increased in MIF during chronic hyperglycemia. These findings are consistent with other reports that established a connection between hyperglycemia or DM with enhanced MMP-9 activity, but not MMP-2, has been shown to be increased in diabetic rats and hyperglycemic bovine aortic EC. Enhanced MMP-9 activity in vitro has been characterized under hyperglycemic conditions, and this increased activity may be reduced by concomitant administration of antioxidants. These intriguing data suggest that reactive oxygen species play an important role in MMP activity. MMP are secreted as proenzymes and are known to be activated by reactive oxygen species. Both DM and hyperglycemia also increase oxidative stress. Taken as a whole, the present and previous data suggest that increased production of reactive oxygen species and the consequent activation of MMP may represent critical factors in the limitation of collateral development during chronic hyperglycemia.

Regulation of Collateral Development
Coronary collateral development is thought to be regulated by the balance of growth factors and growth factor inhibitors. Results in sham dogs support this view and may indicate the presence of “antiangiogenic tone” in dogs that were not subjected to repetitive coronary artery occlusions. However, increases in tube formation in response to angiostatin antibody did not reach statistical significance in sham dogs; thus, this hypothesis will require further investigation to confirm.

In summary, the present results indicate that chronic hyperglycemia inhibits coronary collateral development in dogs. The elimination of collateralization in response to ischemic stimuli may be attributed in part to an increased expression of angiostatin and enhanced MMP-9 activity in the extracellular environment of the collateral-dependent region. The results are consistent with the hypothesis that reduced production of mitogenic and angiogenic substances is not entirely responsible for impaired collateral development. Instead, the present findings provide evidence that enhanced expression of growth inhibitors is a critical contributor to the process. The data suggest that future therapeutic approaches should focus not only on enhanced production or administration of angiogenic proteins but also on localized suppression of angiogenic inhibitors.

Acknowledgments
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References
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