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Prenatal Nicotine Exposure Augments Renal Oxidative Stress in Embryos of Pregnant Rats with Reduced Uterine Perfusion Pressure

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Abstract. *Background/Aim: Both maternal nicotine (NIC) exposure and placental insufficiency increase oxidative stress in the fetal kidney ensuing fetal programming of renal diseases in adult life. Their combined effects, however, are unknown. We tested the hypothesis that maternal NIC exposure exacerbates renal oxidative stress and injury in fetuses of pregnant rats with placental insufficiency. Materials and Methods: Fourteen-day-pregnant rats were subjected to sham operation or reduced uterine perfusion pressure (RUPP) that received either nicotine (20 μg/ml in 1% saccharine) or vehicle (1% saccharine) in their drinking water. At gestational age of 21 days, male fetuses were collected by C-section and sacrificed: plasma and renal cotinine content, extent of renal oxidative stress (4-hydroxynonenal [HNE] and HO-1) and injury (KIM-1) were determined together with the weight of the fetal kidney and fetus. Results: Prenatal NIC exposure resulted in cotinine accumulation in the plasma and kidney of the fetuses, augmented RUPP-associated increase in renal HNE content and HO-1 expression as well as KIM-1 expression. NIC also enhanced RUPP-induced reduction in fetal and fetal kidney weight. Conclusion: Prenatal NIC exposure augments the existing renal risk in the growth-restricted fetus, which may contribute to worsening in fetal programming of renal disease.*

Maternal smoking predisposes the offspring to chronic kidney disease (1, 2). Nicotine (NIC) –the major component of tobacco products, E-cigarettes and NIC replacement therapies– readily crosses the placenta (3), hence, the fetus accumulates significant amounts of NIC (4-6). Nevertheless, NIC accumulation in the fetal kidney is virtually undocumented. Adverse effects of NIC exposure in the kidney depend on generation of reactive oxygen species (ROS) and the resultant oxidative stress (7-9). Maternal NIC exposure increases oxidative stress in the placenta (10) and cord blood of the fetuses (11) as well as in the pancreas (12), microvasculature (13), urine (14) and the kidney (15) of the offspring. It is noteworthy, that the presence of high concentration of NIC exacerbates severity of chronic (16) or acute (8) kidney injury in animal models.

Reduced uterine perfusion pressure (RUPP) –a known model of placental insufficiency– in pregnant rats increases renal oxidative stress in the offspring (17) that results in intrauterine growth restriction (IUGR) and consequent fetal programming of adult chronic kidney disease (18, 19). Whether maternal exposure to NIC increases severity of renal oxidative stress in the IUGR-fetus and consequently augments renal risk for the adult life is completely unknown.

Accordingly, the aim of this study was to reveal whether maternal NIC exposure enhances renal oxidative stress and injury in the embryos of pregnant rats with placental insufficiency induced by reduced uterine pressure.

Materials and Methods

Experimental animals. Timed pregnant Sprague-Dawley rats at gestational day 5 (GD5) (n=8) were divided into four groups as follows: 1./vehicle+sham, 2./ vehicle+RUPP, 3./nicotine+sham and 4./nicotine+RUPP. Vehicle or nicotine administration started on GD5. The dams received either vehicle (1% saccharine) or 20 μg/ml nicotine bitartrate in 1% saccharine as the source of drinking. On GD14 2 groups underwent sham surgery while 2 groups were

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subjected to reduced uterine perfusion pressure (RUPP) surgery, as described elsewhere (17). This procedure induces a 35% to 45% reduction in the blood flow to the fetuses (20). On GD21 the animals were sacrificed: plasma and placentas were collected from the dams; plasma and kidney from the fetuses. Since gender influences the outcome of renal oxidative stress (21) –males being more susceptible (22)- only male fetuses (n=6) were processed for these studies. All these procedures were performed in accordance with guidelines of the Institutional Animal care and use Committee of the University of Mississippi Medical Center.

Plasma and renal cotinine content. Plasma cotinine content –a stable metabolite of nicotine (3)– was determined by a "Cotinine Direct ELISA" kit (Calbiotech, Spring Valley, CA, USA), as recommended by the manufacturer. Renal cotinine content was determined from kidney lysates and normalized to protein content of the lysate.

Determination of renal oxidative stress and injury. Extent of renal oxidative stress was determined from kidney lysates using the OxiSelect HNE-His adduct ELISA kit from Cell Biolabs, Inc. (San Diego, CA, USA). Renal KIM-1 content –a marker of renal tubular injury (23)– was determined from kidney lysates using the Quantikine mouse TIM-1/KIM-1/HAVCR immunoassay kit (R&D Systems, Minneapolis, MN, USA). Values were normalized to protein content of the lysates.

Preparation of lysates and western blotting. Preparation of kidney lysates and western blotting were performed similarly to what we described elsewhere (8). The primary (anti-HO-1) and secondary antibody was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA) and Cell Signaling Technology (Danvers, MA, USA) respectively. Equal loading was determined by rehybridization with actin (Millipore, Billerica, MA, USA). Bands were visualized by Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA), and exposed to an X-ray film (Midwest Scientific, St. Louis, MO, USA). Films were digitized and analyzed by Un-Scan-It Version 6.1 software (Silk Scientific, Orem, UT, USA).

Statistical analysis. Continuous variables are expressed as means and standard deviations (S.D.). One-way ANOVA with Holm-Sidak post-hoc test was used to evaluate differences between groups. Differences between means were considered significant if $p<0.05$. All analyses were performed using the SigmaStat 3.5 (Systat, San Jose, CA, USA) software package.

Results

Cotinine content of the plasma and kidney of fetuses from nicotine-exposed mothers with or without RUPP. Cotinine content –a marker of nicotine exposure– in the plasma and the kidneys of the fetuses from mothers with or without RUPP was determined. As Figure 1 shows, maternal NIC exposure significantly elevated both plasma (Figure 1A) and renal (Figure 1B) cotinine levels compared to the vehicle groups. Interestingly, cotinine levels were higher in the RUPP+NIC group than in the sham+NIC group. It is important to note, that plasma and renal cotinine content in the fetuses were comparable to cotinine levels in maternal plasma and placenta (data not shown).

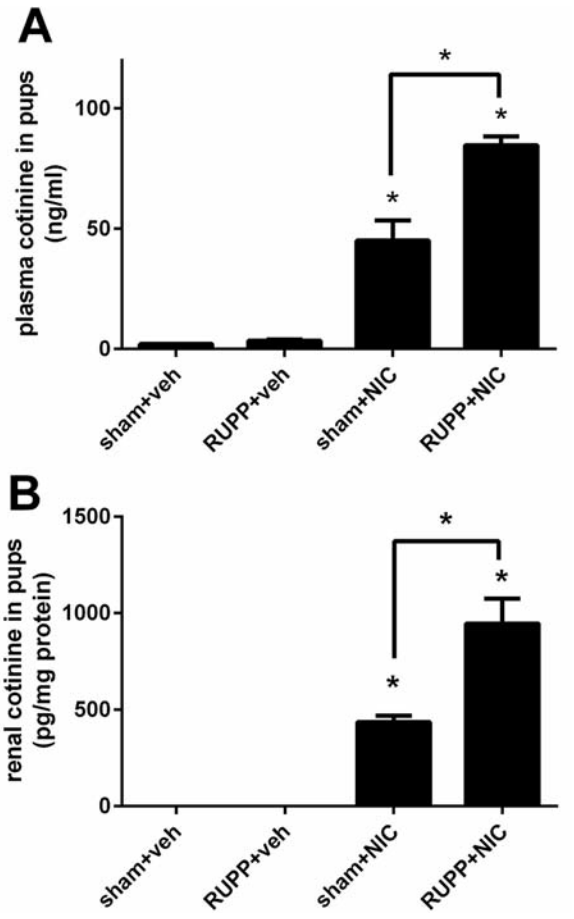

Figure 1. *Effects of maternal nicotine exposure on plasma and renal cotinine content in the fetus from rats with or without reduced uterine perfusion pressure. At day of 14 of gestation (GD14) sham or RUPP surgery was conducted in pregnant rats, as described in the Materials and Methods section. The following day a group of animals received vehicle (1% saccharine) or 20 μg/ml nicotine in 1% saccharine. On GD21 the animals were sacrificed: plasma and kidneys were collected from male pups. (A) Plasma cotinine levels in the pups were determined as described in the Materials and Methods section. n=6, *p<0.05 compared to sham+vehicle or as indicated. (B) Renal cotinine content in the pups' kidney that was normalized to the protein content of the kidney lysates. n=6, *p<0.05 compared to sham+vehicle or as indicated.*

Maternal nicotine exposure exacerbates RUPP-induced renal oxidative stress in the fetuses. Since RUPP enhances renal oxidative stress in the offspring (17) and the presence of nicotine in the kidney is associated with elevated oxidative stress (8), we determined HNE (4-hydroxynonenal) content –a marker of lipid peroxidation and as such tissue oxidative stress– in the kidneys of the fetuses. Figure 2A demonstrates that both RUPP and NIC significantly enhance oxidative stress in the fetal kidneys. Importantly, RUPP-induced

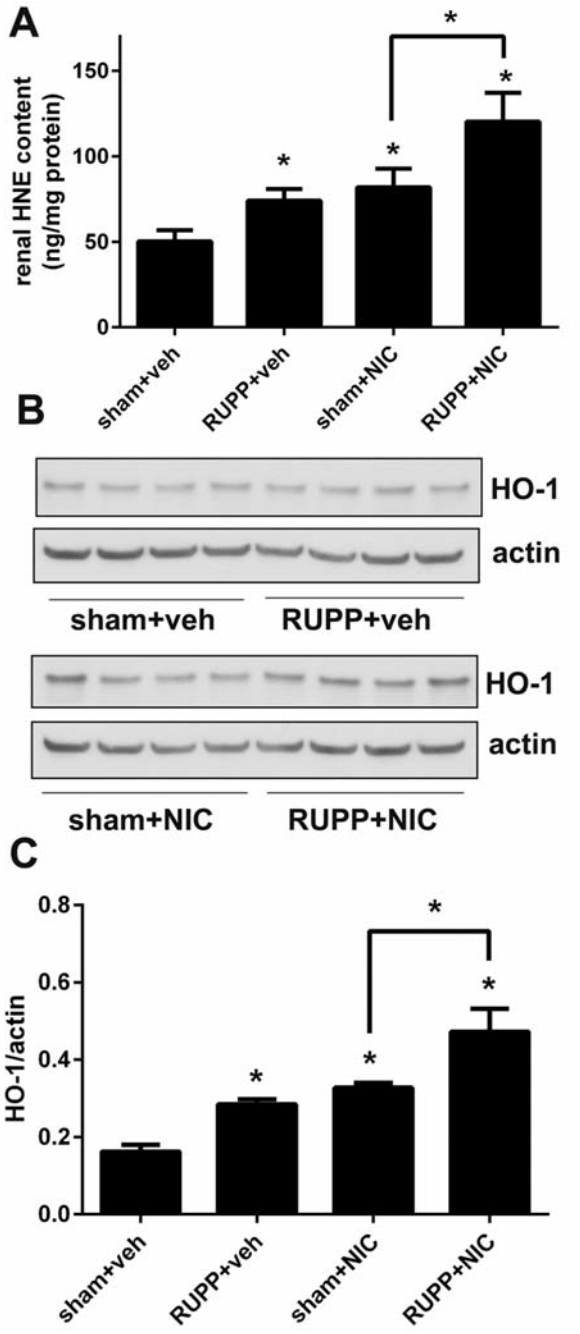

Figure 2. *Prenatal nicotine exposure exacerbates renal oxidative stress in the fetus from rats with reduced uterine perfusion pressure. At GD14 sham or RUPP surgery was conducted in pregnant rats, as described in the Materials and Methods section. The following day a group of animals received vehicle (1% saccharine) or 20 μg/ml nicotine in 1% saccharine. On GD21 the animals were sacrificed: HNE content of the kidneys from male pups was determined and normalized to the protein content of the lysates. n=6, *p<0.05 compared to sham+vehicle or as indicated. (B) Renal expression of HO-1 was determined in kidney lysates of the fetuses by western blotting together with the housekeeping gene actin. Results shown are representative blots from 6 separate animals. (C) Densitometry of the results shown in (B). Results are expressed as HO-1/actin ratios. *p<0.05 compared to sham+vehicle or as indicated.*

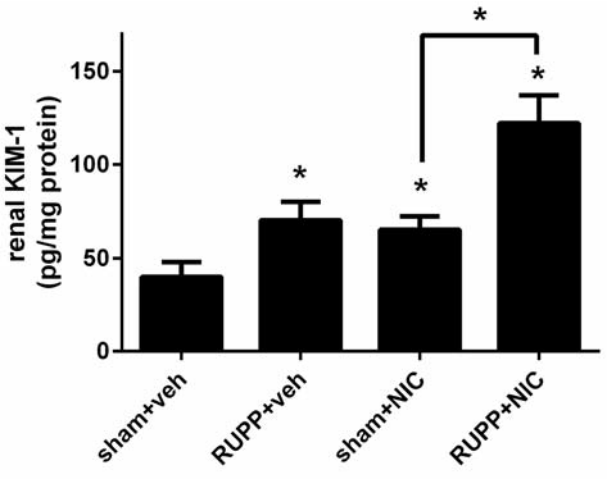

Figure 3. *Prenatal nicotine exposure increases renal injury in fetuses from rats with reduced uterine perfusion pressure. KIM-1 levels were determined in the same renal lysates as described in Figure 2. n=6, *p<0.05 compared to sham+vehicle or as indicated.*

oxidative stress was exacerbated upon maternal NIC exposure. In addition to HNE content, renal expression of the oxidative stress-inducible HO-1 (24) was also determined in fetal kidney lysates by western blotting. As Figure 2B-C shows, both RUPP and NIC significantly induced renal HO-1 and RUPP-mediated induction of HO-1 was further augmented by maternal NIC exposure.

Maternal nicotine exposure augments RUPP-induced increase in expression of KIM-1 in the fetal kidneys. Renal KIM-1 expression –a marker of renal proximal tubular injury– was determined in the fetal kidneys by ELISA. Figure 3 shows that KIM-1 expression exhibits similar pattern to that of oxidative stress: both RUPP and NIC increased it and RUPP-associated increase was further augmented by maternal NIC exposure.

Maternal nicotine exposure and RUPP result in reductions of the fetus, fetal kidney and placental weight. Fetal, fetal kidney and placental weight were recorded on GD21 before delivery. Dams exposed to nicotine (Sham+NIC) show reduced fetal (Figure 4A), fetal kidney (Figure 4B) and placental weight (Figure 4C) compared to Sham+Veh dams. Furthermore, dams underwent RUPP surgery also show reduced fetal (Figure 4A) and placental weight (Figure 4C) comparing to Sham+Veh dams. Importantly, RUPP+NIC significantly decreased fetal, placental and fetal kidney weight comparing to either Sham+NIC or RUPP+Veh. In contrast, placental efficiency (calculated by fetal weight/placental weight) was similar among all groups (Figure 4D).

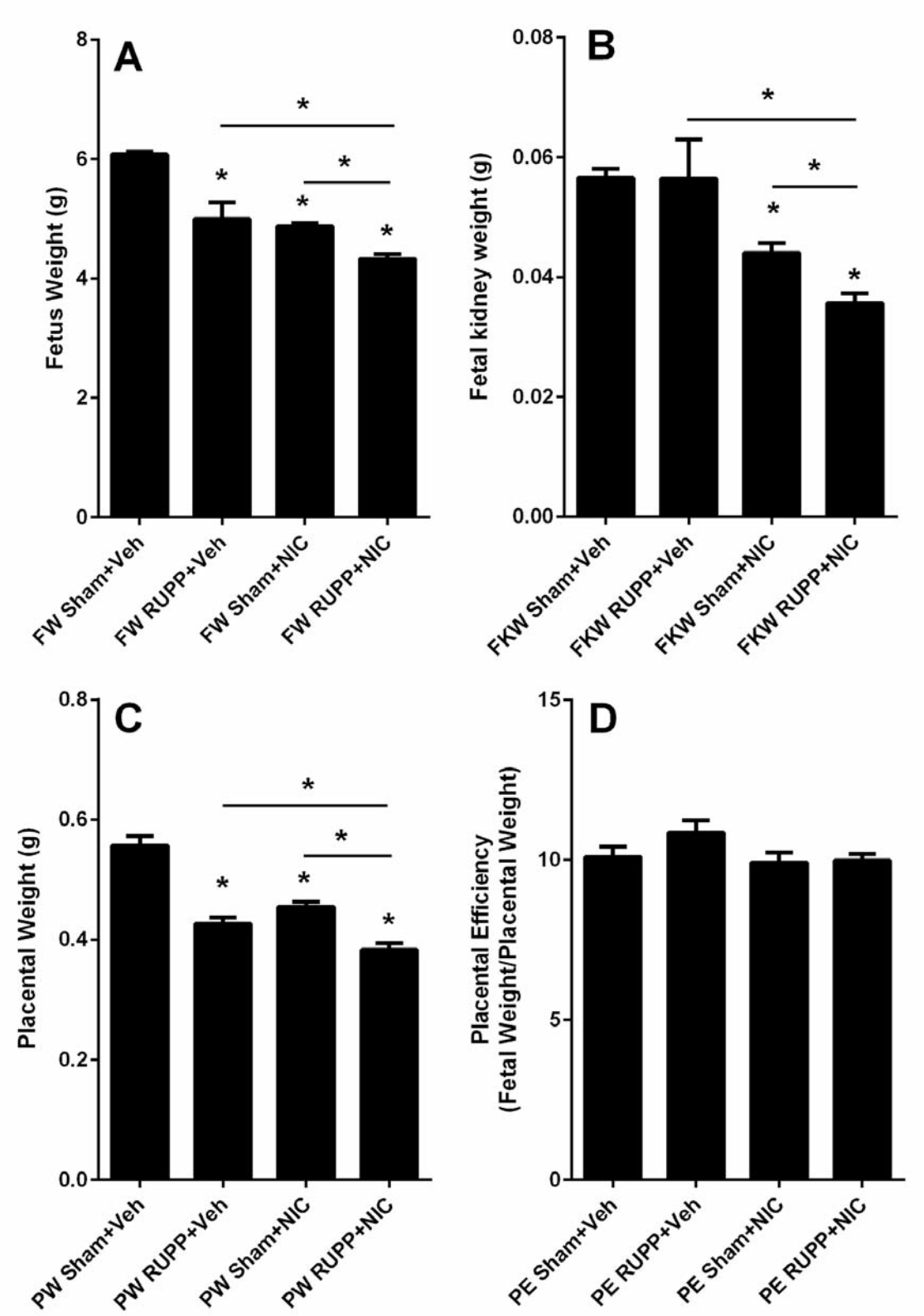

Figure 4. *Impact of maternal exposure to nicotine and reduced uterine perfusion on fetus weight (A), fetal kidney weight (B), placental weight (C) and placental efficiency (D). Weight of fetus, fetal kidney and placenta were recorded immediately after delivery via C-section (n=6). *p<0.05 compared to Sham+Veh or as indicated. FW: Fetus weight; FKW: fetal kidney weight; PW: placental weight; PE: placental efficiency.*

Discussion

Maternal smoking during pregnancy affects the development of the fetal kidney, which can lead to kidney diseases during adult life (25). Studies show that intrauterine nicotine exposure alters renal weight (26), morphology (27) and glomerular mass (28) in the offspring, which are due to increased renal oxidative stress (15). Nevertheless, no data are available on the status of renal oxidative stress in the fetuses that were prenatally exposed to nicotine. We showed that exposing the mothers to nicotine results in high levels of cotinine –a stable nicotine metabolite– in the plasma and kidneys of the fetus (Figure 1), hence, the fetal kidney is at-risk to adverse effects of nicotine during maternal nicotine exposure. Interestingly, RUPP further increased cotinine levels both in the plasma and the kidney of the fetus in NIC-exposed mothers (Figure 1) compared to sham operation. While the mechanism of this phenomenon is unknown, we suggest that this increase might be due to changes in the hemodynamics in the placental-fetal unit leading to reduced blood flow exchange between fetal and placental bi-directional circulation. These changes compromise of nutrient provision and waste removal to and from the fetus and hence, might be nicotine disposal. Certainly, this paradigm warrants further investigation.

Nicotine links smoking to renal injury *via* development of oxidative stress (29). Hence, it is not surprising that the fetal kidneys from NIC-exposed mothers show signs of oxidative stress such as increased levels of HNE and HO-1 (Figure 2). Since RUPP potentiates NIC accumulation in the fetal kidneys (Figure 1B) it may increase renal oxidative stress –at least in part– *via* the increased NIC content. Further studies are needed to evaluate this scenario. Sustained oxidative stress leads to injury: levels of KIM-1 –a marker of renal tubular injury (23)– is elevated in the fetal kidneys upon maternal nicotine exposure (Figure 3). Previously, we showed that chronic exposure to nicotine elevates oxidative stress and KIM-1 in the kidneys of adult mice without altering renal morphology and function (8). Further studies are needed to investigate this scenario in the fetus.

Reduced uterine perfusion pressure (RUPP) is an experimental model of placental insufficiency, which induces intrauterine growth restriction (IUGR) and results in low birth weight offsprings (19). These offspring elicit elevated markers of oxidative stress in the kidneys (17) and increased susceptibility to ischemic renal injury in adult life (19). We found in our study that RUPP increased oxidative stress and tubular injury in the fetal kidneys, which was exacerbated by maternal NIC exposure (Figures 2 and 3). These observations suggest that nicotine exposure and RUPP may have additive effects when applied together. Further studies are also needed to establish whether maternal nicotine exposure affects adverse effects of RUPP in the kidney in adult life. It is a highly plausible scenario as studies, including ours demonstrate, that nicotine exposure exacerbates oxidative stress and consequent injury of the kidney in experimental models of acute (8) or chronic (16, 30) kidney injury.

Additionally, we found that maternal exposure to nicotine reduces weight of the fetus, fetal kidney and placenta, which is even more significant in combination with RUPP (Figure 4A-C). In contrast, placental efficiency did not show differences among groups (Figure 4D), suggesting a compromised placental adaptability to sustain fetal growth resulting in reduced fetal weight.

Conclusion

Our study demonstrated that maternal nicotine exposure increases renal oxidative stress and consequent (probably sub-lethal) injury in the fetus, especially in combination with placental insufficiency. Since E-cigarettes –that contain pure nicotine– are alarmingly popular (31) and they are perceived as a safe alternative to tobacco use (32)–, their use during pregnancy is potentially increasing. These results call attention to the risk of maternal smoking/nicotine exposure in worsening of renal risk in the growth– restricted fetus.

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