Radicicol, a heat shock protein 90 inhibitor, reduces glomerular filtration rate

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Ramírez V, Mejía-Vilet JM, Hernández D, Gamba G, Bobadilla NA. Radicicol, a heat shock protein 90 inhibitor, reduces glomerular filtration rate. Am J Physiol Renal Physiol 295: F1044–F1051, 2008. First published July 30, 2008; doi:10.1152/ajprenal.90278.2008.—The heat shock protein subfamily of 90 kDa (HSP90) is composed of five isoforms. The more abundant proteins of this subfamily are cytosolic isoforms known as HSP90α and HSP90β. More than 100 client proteins have been found to be regulated by HSP90. Several studies have shown that HSP90 regulates nitric oxide synthesis that is dependent on endothelial nitric oxide synthase (eNOS). Because eNOS regulates renal vascular tone and glomerular filtration rate (GFR), the present study was designed to evaluate the effect of acute HSP90 inhibition with radicicol on GFR and the eNOS pathway. Twenty male Wistar rats were divided into two groups: control vehicle animals and radicicol-infused animals (25 μg·ml⁻¹·min⁻¹). Basal levels were taken before experimental measurements. Mean arterial pressure and renal blood flow (RBF) were recorded, as well as GFR, urinary nitrite and nitrate excretion (UNO₂/NO₃,V). Additionally, we evaluated eNOS expression, Ser1177 and Thr495 eNOS phosphorylation levels, the eNOS dimer-to-monomer ratio, as well as oxidative stress by assessing renal lipoperoxidation and urinary isoprostane F₂(ox) and hydrogen peroxide. HSP90 inhibition with radicicol produced a fall in RBF and GFR that was associated with a significant reduction of UNO₂/NO₃,V. The effects of radicicol were in part mediated by a significant decrease in eNOS phosphorylation and in the eNOS dimer-to-monomer ratio. Our findings suggest that GFR is in part maintained by HSP90-eNOS interaction.

Renal blood flow; heat shock protein 90 isoforms

The heat shock protein (HSP) subfamily of 90 kDa is one of the most abundant proteins of eukaryotic cells, comprising 1–2% of total protein under nonstress conditions (30). Five isoforms of HSP90 have been identified, which differ in their cellular localization and abundance. In particular, HSP90α and HSP90β, the major cytoplasmic isoforms, share ~85% sequence identity at protein level. Their main structure encompasses an NH₂-terminal ATPase domain, followed by a charged domain, a client protein binding domain, and a COOH-terminal dimerization domain (26). The ATP binding site is the major target for HSP90 inhibitors, of which geldanamycin, 17-allylamino-17-demethoxy-geldanamycin, and radicicol are the most used because of their high specificity for HSP90 inhibition. These compounds interfere with ATP binding to HSP90, preventing the formation of the mature complex that results in the proteasome-dependent degradation of associated proteins (26).

HSP90 has been shown to interact with and stabilize >100 different client proteins (for a full list see http://www.picard.ch/downloads/Hsp90interactors), including several kinases, transcriptional factors, hormone receptors, antiapoptotic proteins, and, of particular interest for cardiovascular and renal physiology, endothelial nitric oxide synthase (eNOS) (5).

Recent studies have emphasized the physiological role of HSP90 in regulating vascular tone. eNOS is the primary source of nitric oxide (NO) that produces vasorelaxation. It has been demonstrated that HSP90-eNOS coupling increases the activity of this enzyme, with greater NO production as a result. On the contrary, not only is HSP90-eNOS uncoupling associated with reduction of NO synthesis but it also turns eNOS into a superoxide generator (10, 24, 34). These studies strongly suggest that the dual role of eNOS in vascular physiology is modulated by its interaction with HSP90. Despite the growing evidence that this interaction is relevant for vascular physiology, little to nothing is known about the role that HSP90 plays in renal physiology. In this regard, we previously (27) characterized the expression pattern of HSP90α and HSP90β along the nephron, observing that both proteins are expressed in glomerular capillaries, mesangial cells, and Bowman epithelia and along tubular epithelium. Although it is well known that eNOS is highly expressed in renal vascular endothelial cells, and that renal blood flow (RBF) and glomerular filtration rate (GFR) are regulated by eNOS-dependent NO production, it is unknown to what extent RBF and GFR depend on HSP90-regulated eNOS activity. Here we show that HSP90 inhibition with radicicol results in a significant reduction of RBF and GFR that is associated with decreased NO generation. These findings suggest that HSP90 is an important regulator of renal function.

MATERIALS AND METHODS

Twenty male Wistar rats weighing 300–320 g each and fed with a standard chow diet were divided into two groups: control rats and rats in which HSP90 was acutely inhibited by radicicol infusion (see below). All experiments were performed in a control period and a vehicle or radicicol infusion period. Animal procedures were in accordance with our institutional guidelines for animal care. All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington DC, 1996) and were approved by Institutional Animal Care and Use Committee of the National University of Mexico.

Functional studies. The day of the experiment, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37°C. The trachea, jugular vein, and femoral artery were cannulated with polyethylene tubing (PE-240 and PE-50). The bladder was also cannulated with PE-90. During surgery, rats were

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maintained under euvoletic conditions by infusion of 10 ml/kg body wt isotonic rat plasma, followed by an infusion of 5% low-caloric commercial sugar (METCO, Mexico City, Mexico) at 1.6 ml/h as a marker of GFR. We previously showed (23) that this compound has enough sensitivity to measure GFR under normal and pathophysiological conditions to a similar extent as the standard measurement using polyfructosan. All experiments were performed in two stages. In the first stage, after an appropriate equilibrium period of 60 min urine was drained from the bladder by gravity. Care was taken to avoid dead space in the bladder while urine was collected over a period of 30–60 min. Blood samples were taken at the beginning and end of each urine collection period and replaced with blood from a donor rat. An ultrasound transit-time flow probe (1RB, Transonic, Ithaca, NY) was placed around the left renal artery and filled with ultrasonic coupling gel (HR Lubricating Jelly, Carter-Wallace, New York, NY) for recording RBF. Mean arterial pressure (MAP) was monitored throughout the experiment with a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA). After basal measurements, the second stage began. In addition to low-calorie sugar infusion, one-half of the rats received an infusion of 10% DMSO and 10% ethanol in saline solution as a vehicle. The other half received an infusion of the HSP90 inhibitor radicicol (25 μg/kg; 10% DMSO and 10% ethanol in saline solution) (22). After 45 min of equilibrium, all measurements of GFR, RBF, urine flow, and MAP were repeated. At the end of the experiment, both kidneys were removed and quickly frozen for biochemical and molecular studies.

To confirm our results with low-calorie commercial sugar, an additional group of six rats was included in which renal function before and after radicicol was evaluated by using 5% polyfructosan as a gold standard GFR marker (Inutest, Laevosan, Linz, Austria).

Low-calorie sugar concentrations in urine and plasma were determined by the technique of Davidson and Sackner (8) for determining GFR. Low-calorie sugar clearance was calculated by standard formula as we previously reported (23).

Urinary nitrites and nitrate excretion. The end products of NO, nitrites and nitrates (NO2− and NO3−), were estimated in 30-min urine samples by reducing NO2− to NO3− with nitrate reductase (Roche) and β-adenine nicotinamide (β-NADPH, Sigma), followed by nitrite quantification with the Griess reagent, as we (21) and others (6) previously reported.

Renal lipoperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) as previously described (20). Briefly, after homogenization of the renal tissue, the reaction was performed in a 0.8% aqueous solution of thiobarbituric acid in 15% TCA and heated at 95°C for 45 min. TBARS were quantified with an extinction coefficient of 1.56 × 10^5 M^-1 cm^-1 and expressed as nanomoles of TBARS per milligram of protein. The tissue protein was estimated with the Bradford method.

Urinary hydrogen peroxide and isoprostane F2αx assays. The amount of hydrogen peroxide (H2O2) in urine was determined with an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to manufacturer’s instructions. Briefly, the assay was performed with a standard curve of H2O2, 1–10 μM. A 50-μl volume of each urine or standard was placed in a microplate, 50 μl of Amplex red reagent-horseradish peroxidase (HRP) was then added, and the samples were incubated for 30 min at room temperature and protected from light. The plate was read at 560 nm. The H2O2 concentration in the samples was expressed as nanomoles per milliliter.

The concentration of isoprostanes F2αx (8-iso-PGF2αx) were determined in urine samples with a urinary 8-iso-PGF2αx ELISA assay from Northwest (Vancouver, WA) according to the manufacturer’s instructions. Briefly, 100 μl of each sample or standard was placed in wells that contained the anti-8-iso-PGF2α antibody, and 100 μl of 15-isoprostane F2T HRP enzyme conjugate was added. Then 200 μl of 3′3′5′5′-tetramethyl benzidine substrate was added to the wells and incubated again; the reaction was stopped by addition of 50 μl of 3 M H2SO4, and the wells were read at 450 nm. The isoprostane F2αx concentration was reported as nanograms per milliliter.

RNA isolation and real-time PCR. The renal cortex was isolated from both kidneys and snap frozen in liquid nitrogen. Total RNA was isolated from each kidney with the TRIzol method (Invitrogen) and checked for integrity by 1% agarose gel electrophoresis. To avoid DNA contamination, all total RNA samples were treated with DNase (Invitrogen). Reverse transcription (RT) was carried out with 2.5 μg of total RNA and 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The mRNA levels of HSP90α and HSP90β as well as eNOS were quantified by real-time PCR on the ABI Prism 7300 Sequence Detection System (TaqMan, ABI, Foster City, CA). Primers and probes for HSP90α and eNOS were ordered as kits: Rn00822023-g1 and Rn02132634-s1 (Assays-on-Demand, ABI) and HSP90BRAT-X (Assay-on-Design, ABI) for HSP90β. As an endogenous control, we used eu karyotic 18s rRNA (designed assay reagent Applied by ABI, external run). Relative quantification of HSP90α, HSP90β, and eNOS gene expression was performed with the comparative threshold cycle (CT) method (18).

Western blot analysis. Total renal proteins were isolated from six different cortices of each group and homogenized separately in lysis buffer (50 mM HEPES pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, and Complete protease inhibitor (Roche)). Protein samples containing 50 μg of total protein were resolved by 7.5% SDS-PAGE, semidried, and electroblotted onto polyvinylidene difluoride membranes (Amersham). eNOS protein (Abcam, Cambridge, MA) or HSP90α transfected HEK cells were loaded as positive controls. Membranes were then blocked first with 0.1% bloting grade blocker nonfat dry milk (Bio-Rad) and incubated in 5% bloting grade blocker nonfat dry milk with their respective specific antibodies as detailed below. The lower part of the membranes was incubated with a goat anti-actin antibody (1:5,000 dilution) overnight at 4°C (Santa Cruz Biotechnology, Santa Barbara, CA). The upper membranes were incubated with polyclonal anti-rabbit HSPB4 (HSP90β, Abcam), polyclonal anti-rabbit HSPB6 (HSP90α, Abcam), anti-rabbit eNOS (Cell Signaling Technology), polyclonal anti-rabbit phosphorylated eNOS-S1177 (Cell Signaling Technology), or polyclonal anti-rabbit phospho-ENOST495 antibodies (Cell Signaling Technology). Membranes were then incubated with the secondary antibody HRP-conjugated rat anti-rabbit IgG (Alpha Diagnostics, San Antonio, TX). Proteins were detected with an enhanced chemiluminescence kit (Amersham) and autoradiography, following the manufacturer’s recommendations.

The eNOS dimer-to-monomer ratio was evaluated by Western blot in nonnated proteins isolated from cortices of both vehicle- and radicicol-infused animals as previously reported (4, 14). Nonboiled samples containing 50 μg of total protein were resolved in a 6% SDS-PAGE at 4°C. Proteins were transferred to a polyvinylidene difluoride membrane, and Western blot analysis for enOS was performed as described above. The bands were scanned for densitometric analysis.

Statistical analysis. Results are presented as means ± SE. Differences between control and experimental periods in the same group were tested by paired t-test. Significant differences among the groups were tested by ANOVA with Bonferroni’s correction for multiple comparisons. Statistical significance was defined when the P value was <0.05.

RESULTS

We evaluated the role of HSP90 in regulating renal function by acutely inhibiting HSP90 in rats with radicicol. Renal function was evaluated in each animal in two steps: during normal euvoletic conditions and during vehicle or radicicol infusion. All animals had similar body weight (control animals 303 ± 3.1 g, radicicol-infused animals 309 ± 2.8 g). Radicicol infusion produced a significant reduction in RBF, from 8.6 ± 1.1 to 5.6 ± 0.3 ml/min (P = 0.01), which represents a
HSP90 inhibition reduces GFR

Inhibition, we first evaluated whether the expression of HSP90 and HSP90β remained unaltered after acute HSP90 inhibition. Radicicol did not modify HSP90α and HSP90β mRNA levels in renal cortex compared with the expression observed in vehicle-infused animals (Fig. 2, A and B). Accordingly, no differences were found in HSP90α and HSP90β protein levels between groups (Fig. 2, C and D).

To investigate whether renal vasoconstriction induced by HSP90 inhibition by radicicol was mediated by either reduced NO availability or increased generation of reactive oxygen species, we evaluated urinary nitrite and nitrate excretion (UNO2/NO3V), renal lipoperoxidation, and the amount of isoprostane F2α and hydrogen peroxide in the urine. As shown in Fig. 3A, radicicol infusion produced a significant decrease in urinary NO metabolites by 58%. This effect was not observed in the animals that received vehicle infusion. At the end of the experiment, renal lipoperoxidation was measured in the cortex and medulla separately (Fig. 3B). In contrast to the effect on NO metabolite excretion, HSP90 inhibition did not modify MDA values compared with those in vehicle-infused animals. In addition, greater lipoperoxidation levels were observed in the medulla than in the cortex in both groups. These findings could be expected because of the higher hypoxia in the medullary region. To confirm the absence of change in oxidative stress rate, more specific assays, such as isoprostanes and hydrogen peroxide, were used. Figure 3, C and D, show that HSP90 inhibition did not modify urinary hydrogen peroxide and isoprostane F2α, respectively, confirming our observations at the tissue level. Together, these results suggest that the observed GFR reduction induced by acute radicicol infusion was, in part, mediated by a decrease in NO generation rather than an increase in oxidative stress.

To evaluate whether NO reduction by radicicol was mediated by changes in eNOS expression and phosphorylation, we evaluated renal eNOS mRNA and protein levels by real-time PCR and Western blot. We also evaluated Ser1177 and Thr497 eNOS phosphorylation in the renal cortex of vehicle- and radicicol-infused animals by using phospho-specific antibodies against each site. We found that eNOS mRNA levels were not modified by either vehicle or radicicol infusion (Fig. 4A). Similar results were observed when eNOS protein levels were

![Fig. 1. Effect of heat shock protein (HSP90) inhibition on renal function. A: mean arterial blood pressure (MAP). B: mean renal blood flow (RBF) recorded by a renal artery ultrasonic probe. C: mean glomerular filtration rate (GFR) evaluated with a GFR marker. Open bars, basal (B) period before vehicle or radicicol infusion; gray bars, vehicle (Veh) infusion period; filled bars, radicicol (Rad) infusion period. Statistical differences between basal and experimental periods were tested by paired t-test; *P < 0.05.](http://ajprenal.physiology.org/)

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assessed, as represented by Western blot and densitometry analysis (Fig. 4B). In contrast, a significant increase in eNOS phosphorylation at Thr495 was observed. The immunodetection signal of eNOS phosphorylated at Thr495 was increased in rats infused with radicicol (Fig. 4C). The mean ratio of eNOS phosphorylated at Thr495 to actin during HSP90 inhibition was 0.78 ± 0.12 compared with 0.47 ± 0.18 in vehicle-infused animals (P < 0.02). In contrast, radicicol infusion did not produce any effect on Ser1177 eNOS phosphorylation (Fig. 4D). The mean ratio of eNOS phosphorylated at S1177 to actin was similar in vehicle- and radicicol-infused rats (0.59 ± 0.14 and 0.57 ± 0.13, respectively). Since eNOS expression levels did not change by radicicol infusion, the ratios of pThr495-eNOS/eNOS and pS1177-eNOS/eNOS.

It has also been demonstrated that eNOS activation requires the formation of its dimeric form since monomers exhibit significantly lower activity (1, 15). In this study, we also evaluated whether HSP90 inhibition modified the ratio of the monomeric vs. the dimeric state of eNOS. We observed that HSP90 inhibition was associated with a significant increase in monomeric eNOS together with a reduction in the dimeric form when proteins were analyzed under nondenatured conditions (Fig. 5A). This was confirmed by densitometric analysis of the bands corresponding to dimeric and monomeric eNOS forms in the renal cortex (Fig. 5, B and C). As a result, the ratio between the dimeric and monomeric forms was significantly reduced, from 1.74 ± 0.32 in vehicle-infused rats to 0.33 ± 0.07 in radicicol-infused rats (Fig. 5D; P < 0.05).

DISCUSSION

In this study, we have shown that infusion of the HSP90 inhibitor radicicol produced a significant decrease in RBF and GFR, which was associated with a decrease in the excretion of NO metabolites in the urine. Reduction in NO synthesis seems to be a consequence of an increase in inactivating eNOS phosphorylation and the concomitant increase in eNOS monomerization. Thus our results suggest that HSP90 is an important modulator of GFR by favoring the renal NO/eNOS system.

Molecular chaperones can assist in the folding and maintenance of newly translated proteins, activate proteins through promoting their phosphorylation or protein-protein interactions, and also lead to degradation of misfolded and destabilized proteins. Indeed, HSP90 is a key member of this machinery. It plays a central role in cellular signaling since it is essential for maintaining the activity of several signaling proteins, including steroid hormone receptors and enzymes such as eNOS.

Previous studies have shown that the association of HSP90 with eNOS plays a key role in the generation of NO through several mechanisms, including enhancing eNOS enzymatic activity, modulating the release of NO vs. superoxide by eNOS, assisting with the intracellular trafficking of eNOS, and helping to activate eNOS by dissociating it from caveolin-1 (16). Furthermore, HSP90 has been proposed to facilitate the calcineurin-dependent dephosphorylation of the Thr495 site of eNOS, which contributes to enzyme activity (16). In this study, we found that the acute infusion of radicicol, an HSP90 inhibitor, produced decreases in RBF and GFR that were associated with a significant decrease in the urinary excretion of NO metabolites (UNO2/NO3V), suggesting that eNOS-HSP90 uncoupling resulted in reduced NO synthesis that, in turn, promoted renal vasoconstriction.

In support of this observation, recent findings by Xu et al. (34) demonstrated that disruption of the eNOS-HSP90 complex with decoy peptides that block the interaction between HSP90 and eNOS inhibited eNOS-dependent NO generation and reduced acetylcholine-
induced vasodilation in mouse facialis arteries. In addition, it has been shown that inhibition of HSP90 not only decreases stimulated NO synthesis but also increases superoxide anion generation, which has been shown to be dependent on eNOS (10, 24, 34). These reports indicated that inhibiting eNOS-HSP90 coupling shifts the product of eNOS from NO to O2

In this regard, we observed that acute HSP90 inhibition was associated with decreased NO synthesis rather than increased oxidative stress that was measured by renal lipoperoxidation and urinary H2O2 and isoprostanes F2

, suggesting that renal vasoconstriction was in part due to the reduction of NO availability, although other pathways regulated by HSP90 might be involved, considering that several proteins are regulated by HSP90. In this regard, it has been shown that HSP90 also activates neuronal nitric oxide synthase (nNOS) (2, 29). NO derived from nNOS, present in macula densa cells, might regulate glomerular blood flow and GFR by modulating afferent arteriolar vascular tone (for review, see Ref. 33); thus it is also plausible that HSP90 inhibition could inactivate nNOS and this effect could be also involved in the observed reduction of GFR. Further experiments with specific nNOS inhibitors or nNOS-null mice will be required to clarify this issue. However, in the present study we observed several changes in eNOS-NO pathway, suggesting that at least part of the radicicol effect must be due to its effect on eNOS.

To investigate the mechanism by which HSP90 inhibition produced a decrease in NO metabolites, we evaluated eNOS phosphorylation and dimerization. Several putative eNOS phosphorylation sites have been reported. The most studied are the Ser1177 site, which activates eNOS, and the Thr495 site, which inactivates it. This threonine residue is found in the calmodulin (CaM) binding domain and interferes with CaM binding, resulting in the inactivation of eNOS (19, 31). In this regard, coupling of HSP90 and eNOS is associated with enhanced Ser1177 phosphorylation by Akt (32), whereas uncoupling of eNOS-HSP90 promotes Thr495 phosphorylation by protein kinase C (19). In this study, we observed that radicicol infusion did not alter Ser1177 phosphorylation but was able to increase Thr495 phosphorylation without changing eNOS protein levels. This finding suggests that reduced NO synthesis by HSP90 inhibition is at least due to reduction of eNOS activity that follows augmented Thr495 phosphorylation.

Not only is activation of eNOS dependent on phosphorylation state by upstream kinases, but it is also determined by its conformational state (13). It is known that NO is produced by eNOS when it is conformed as a homodimer. This conformation creates high-affinity binding sites for the NOS substrate L-arginine, enabling electron transfer from the reductase domain of one NOS monomer to the oxygenase domain of the other monomer (28). Interestingly, we observed that acute infusion of radicicol produced a significant reduction of the dimer-to-monomer ratio due to an increase in eNOS monomers. This observation suggests that, in addition to eNOS phosphorylation at the Thr497 site, inhibition of HSP90 with radicicol reduces NO production by preventing the formation of eNOS homodimers.

The physiological relevance of eNOS-HSP90 uncoupling has been recently attested in experimental models of disease, such as diabetes mellitus, arterial hypertension, and liver cirrhosis. Diabetes is characterized by NO deficiency. Lei et al. (17) demon-
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The arterial hypertension that occurs during pregnancy known as preeclampsia is, in part, mediated by an increase in endothelial oxidative stress in maternal vasculature. To explain the mechanism, Gu et al. (12) demonstrated that HSP90 protein expression was significantly decreased in endothelial cells from preeclamptic women, which corre-

Fig. 5. Effect of HSP90 inhibition on the expression of eNOS protein dimer/monomer. A: representative image of a Western blot analysis of nondenatured proteins isolated from cortexes of vehicle control and radicicol-treated rats. B–D: dimeric eNOS (B), monomeric eNOS (C), and eNOS dimer-to-monomer ratio (D/M; D) in vehicle (open bars) and radicicol-infused (filled bars) animals. Statistical differences between groups were tested by unpaired t-test; *P < 0.05. OD, optical density.
lated with greater superoxide generation. In addition, a more recent study reported that hypertension and endothelial dysfunction induced by increased production of 20-hydroxyeicosatetraenoic acid (20-HETE), a major vasoconstrictor eicosanoid in the microcirculation, were in part due to eNOS-HSP90 uncoupling (7).

Finally, another pathophysiological role for eNOS-HSP90 coupling/uncoupling was observed in rats with hypertensive syndrome, a complication of cirrhosis that is characterized by overproduction of NO in lungs and NO deficiency in the liver. In an elegant study, Frossard et al. (9) showed an opposite regulation of eNOS by HSP90 since greater NO synthesis in the lungs was associated with elevated eNOS and HSP90 interaction, whereas the contrary effect was observed in liver homogenates, in which NO production was decreased. Thus lesser NO production in the liver was attributed to eNOS-HSP90 uncoupling together with enhanced association of eNOS to the caveolin. Together, these studies indicate that HSP90-eNOS coupling/uncoupling ratio may play a major role in several pathophysiological conditions. Thus it will be interesting to study the role that HSP90-eNOS coupling/uncoupling could play in the progression of kidney diseases, such as diabetic or hypertensive nephropathy, in which renal vascular dysfunction plays a major role.

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