Attenuated vasoconstrictor responses to endothelin in afferent arterioles during a high-salt diet

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Schneider MP, Inscho EW, Pollock DM. Attenuated vasoconstrictor responses to endothelin in afferent arterioles during a high-salt diet. Am J Physiol Renal Physiol 292: F1208–F1214, 2007. First published January 9, 2007; doi:10.1152/ajprenal.00280.2006.—Endothelin-1 (ET-1) is increased in rats on a high-salt (HS) diet and participates in salt-dependent hypertension. Afferent arterioles (AA) are important for long-term blood pressure control, and therefore we hypothesized that a HS diet would alter their responsiveness to ET-1. Sprague-Dawley rats were fed either a normal-salt (NS; 0.66% NaCl) or HS (8%) diet for 1 wk. Diameters of AA were determined in response to increasing concentrations of big ET-1, ET-1, sarafotoxin 6c (S6c), or norepinephrine (NE), using the blood-perfused juxtamедullary nephron technique. ET-1 responses were also determined during blockade of endothelin type A (ETA) or type B (ETB) receptors with 10 nM ABT-627 or 30 nM A-192621, respectively. Expression of ETA and ETB receptors was determined in renal microvessels. Responses of AA to big ET-1, ET-1, and S6c were significantly attenuated during a HS diet (e.g., response to 10–10 M ET-1 in NS vs. HS rats: −52.5 ± 10.2 vs. +5.6 ± 11.3% of control diameter; P < 0.05), with no change in the responses to NE. ETB, but not ETA receptor blockade abolished the different response to ET-1 between the NS and HS groups. ETB receptor expression in renal microvessels was increased in response to HS (17.7 ± 2.4 vs. 6.6 ± 3.0% of β-actin, P = 0.02), whereas ETA receptor expression was unchanged. These results suggest that the reduced vasoconstrictor response of AA to endothelin peptides during a HS diet is mediated by increased vasodilatory function of endothelial ETB receptors. By preserving renal blood flow, this may be an important mechanism to restore sodium balance during a HS diet.

endothelin-1; sodium; renal circulation; hypertension

ENDOTHELIN-1 (ET-1), one of the most potent vasoconstrictors in humans known today, is produced by the vascular endothelium and acts on two types of receptors. The endothelin type A (ETA) receptor is located on smooth muscle cells and mediates long-lasting vasoconstriction (17, 37). Some endothelin type B (ETB) receptors are also located on smooth muscle cells, contributing to ET-1-induced vasoconstriction (17, 37). ETB receptors are also present on endothelial cells, which limits vasoconstrictor activity by the release of nitric oxide and by clearing ET-1 from the circulation (10, 39). Since its discovery, involvement of the endothelin system has been documented in a broad spectrum of cardiovascular conditions, such as hypertension, heart failure, renal failure, and pulmonary hypertension.

More recently, there has been considerable interest in the role of endothelin in the regulation of kidney function. Interestingly, the intrarenal endothelin system is an important mediator of natriuresis, which contrasts with the overall prohypertensive effects of ET-1 in the vasculature. Inner medullary collecting duct (IMCD) cells produce high levels of ET-1 (24), which act locally on ETB receptors to inhibit tubular sodium transport and promote natriuresis (25, 30). Pharmacological blockade of ETB receptors, or disruption of the ETB receptor gene, produces a salt-sensitive phenotype (11, 33). Recent data show that tissue-specific knockout of the ET-1 gene in IMCD cells results in salt-sensitive hypertension in mice (1). Furthermore, medullary ETB receptor expression is upregulated in DOCA-salt hypertension (31), and the ETB receptor mediates an increased medullary blood flow response to big ET-1 in rats fed a high-salt (HS) diet (38). These data indicate an important role for this intrarenal signaling pathway in adjusting sodium excretion to match variations in dietary salt intake.

In contrast to the effects of ET-1 in the renal medulla, the regulation of the endothelin system in the renal vasculature by dietary sodium has not been examined. Afferent arterioles are highly specialized vessels, with profound impact on renal vascular resistance and renal blood flow. Furthermore, increases in afferent arteriolar resistance can lead to the development of arterial hypertension (14). ET-1 is a powerful vasoconstrictor of afferent arterioles (9, 23). Interestingly, it has been demonstrated that ET-1-mediated vasoconstriction of mesenteric arteries is enhanced in rats fed a HS diet (4). Because of the importance of renal vascular tone for renal function and long-term blood pressure control, we examined the effects of a HS diet on the response of afferent arterioles to endothelin peptides. In addition to these functional studies, we also determined whether a HS diet influenced the expression of ETA and ETB receptors in renal preglomerular microvessels.

METHODS

All experiments were performed with approval from the Institutional Animal Care and Use Committee at the Medical College of Georgia. Male Sprague-Dawley rats (275–300 g body wt, Charles River Laboratories, Wilmington, MA) were divided into two groups. One group received a normal-salt (NS) diet (0.66% NaCl, Harlan Laboratories, Indianapolis, IN), while the other group received a HS diet (8% NaCl). Tap water was given ad libitum. After 1 wk, videomicroscopic experiments were performed using the juxtamedullary nephron technique and preglomerular microvessels were isolated for immunoblot analyses.

Measurement of blood pressure. Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (ITC, Woodland Hills, CA) before and after 1 wk of either an NS or HS diet.
are no selective ETA receptor agonists available, ET-1 responses were
function using a second approach by repeating the responses to ET-1
continuously in a closed reservoir while being oxygenated with a 95%
microvasculature on the inner cortical surface was isolated by tying
microvasculature of juxtamedullary nephrons. A portion of the renal
mucosa was removed to expose the renal tubules, glomeruli, and
donor rat. The perfused kidney was removed and sectioned along the
also determined in the presence of the ET B receptor blocker,
vided by Abbott Laboratories (North Chicago, IL).

Increasing concentrations of NE ranging from 10 nM to 1 μM were
used to examine whether any alterations of the afferent arteriolar
response during HS are specific to the endothelin system. Measure-
ments of arteriolar diameters were made at 12-s intervals, and the
sustained diameter was calculated from the average of measurements
made during the final 2 min of each treatment period. Each protocol
consisted of six to nine 5-min periods.

Isolation of renal microvessels. Renal microvessels were isolated
according to a method described previously (40). Briefly, the kidneys
were perfused with a physiological salt solution containing 1% Evans
blue, and the renal microvessels were separated from the rest of the
cortex with the aid of sequential sieving, a digestion period, and
collection under a stereomicroscope. Renal microvessels were col-
lected, quickly frozen in liquid nitrogen, and kept frozen (−80°C)
until use in Western blot analysis.

Immunoblot analysis of ETA and ETB receptor protein. Homoge-
nenates of renal microvessels were separated by electrophoresis on a
10% stacking Tris-glycine gel, and proteins were transferred electrop-
oretically to a nitrocellulose membrane. The primary antibodies
used were rabbit anti-rat ETA receptor polyclonal antibody (1:1,000;
Fitzgerald Industries International, Concord, MA) and rabbit anti-rat
ETB receptor polyclonal antibody (1:200; Alomone Labs, Jerusalem,
Israel). The blots were washed in phosphate-buffered saline-0.1%
Tween 20 and incubated with the secondary antibody (goat anti-rabbit
1:5,000 for the ETA; 1:15,000 for the ETB receptor) conjugated to
horseradish peroxidase for 1 h at room temperature and washed.
Detection was accomplished by enhanced chemiluminescence West-
ern blotting (ECL, Amersham Biosciences, Pittsburgh, PA), and blots
were exposed to X-ray film (Hyperfilm-ECL, Amersham Biosciences).
Band intensity was measured densitometrically, and the
values were factored for β-actin (Sigma).

Statistics. All data are presented as means ± SE. The effect of HS
vs. NS treatment and the effect of the vasoactive agents on afferent
arteriolar diameters were analyzed by two-way ANOVA. Subsequent
Bonferroni analysis was performed to compare afferent arteriolar
diameters between the two treatment groups at single concentrations
of the various vasoactive agents. An unpaired two-tailed t-test was
applied to compare the ETA and ETB receptor protein levels deter-
mined by densitometry. A value of P < 0.05 was considered
statistically significant.

RESULTS

SBP was similar after 1 wk on either NS or HS diet and
averaged 111 ± 5 and 110 ± 5 mmHg, respectively. In
addition, baseline afferent arteriolar diameters were similar
between the NS and HS groups before any of the subsequent
protocols were performed (Table 1).

Afferent arteriolar response to big ET-1 during NS and HS.
In the NS group, big ET-1 produced vasoconstriction at a
concentration of 10−9 M and higher (P = 0.02 for 10−9 M
compared with baseline; Fig. 1). At the highest concentration,
10−7 M, a contraction of −46.3 ± 4.5% of baseline vessel

<table>
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<th>Protocol</th>
<th>NS</th>
<th>HS</th>
<th>P Value</th>
</tr>
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<tr>
<td>Big ET-1</td>
<td>16.8±2.5 (n=4)</td>
<td>17.1±1.1 (n=6)</td>
<td>NS</td>
</tr>
<tr>
<td>ET-1</td>
<td>18.5±0.6 (n=6)</td>
<td>17.6±1.2 (n=6)</td>
<td>NS</td>
</tr>
<tr>
<td>S6c</td>
<td>17.9±1.2 (n=6)</td>
<td>16.8±1.3 (n=6)</td>
<td>NS</td>
</tr>
<tr>
<td>ET-1+A-192621</td>
<td>16.7±1.8 (n=5)</td>
<td>17.5±1.1 (n=4)</td>
<td>NS</td>
</tr>
<tr>
<td>NE</td>
<td>19.4±1.1 (n=6)</td>
<td>18.9±0.8 (n=6)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE, n, No. of rats; NS, normal salt; HS, high salt; ET-1, endothelin-1; S6c, sarafotoxin 6c; NE, norepinephrine; NS, not significant.

Table 1. Baseline afferent arteriolar diameters in rats treated with NS and HS diets for 1 wk
diameter was achieved ($P = 0.002$). In contrast, in the HS group, big ET-1 caused vasodilation from the lowest concentration of $10^{-12}$ M ($P = 0.01$ compared with baseline) to a concentration of $10^{-10}$ M ($P = 0.03$). The afferent arteriolar response reverted to vasoconstriction from $10^{-9}$ to $10^{-8}$ M [both not significant (NS) compared with baseline]. At the highest concentration of $10^{-7}$ M, a contraction of $-47.7 \pm 12.6\%$ of baseline diameter was achieved ($P = 0.01$). The vascular responses to big ET-1 were significantly different between the two groups, resulting in a rightward shift in the concentration-response relationship.

**Afferent arteriolar response to ET-1 during NS and HS.** In vessels from rats on a NS diet, ET-1 produced afferent arteriolar vasoconstriction at concentrations of $10^{-11}$ M and higher ($P = 0.05$ for $10^{-11}$ M compared with baseline; Fig. 2), resulting in a more potent effect compared with big ET-1. At the highest applied concentration of $10^{-8}$ M, ET-1 reduced diameter by $-80.5 \pm 0.6\%$ compared with baseline diameter ($P < 0.0001$ compared with baseline). In the HS group, ET-1 caused vasodilation at the lower concentrations of $10^{-12}$ to $10^{-11}$ M (both $P = 0.05$). The vascular response reverted from vasodilation to vasoconstriction at the subsequent concentrations from $10^{-10}$ to $10^{-9}$ M (NS compared with baseline). The highest concentration of $10^{-8}$ M reduced diameter by $-68.5 \pm 11.1\%$ compared with baseline diameter ($P = 0.004$). The afferent arteriolar responses to ET-1 were significantly different between the two groups, resulting in a rightward shift in the concentration-response relationship for vessels from rats treated with a HS diet ($P < 0.0001$).

**Afferent arteriolar response to S6c during NS and HS.** In vessels from animals on a NS diet, S6c constricted afferent arterioles at concentrations of $10^{-10}$ M and higher ($P = 0.05$ for $10^{-10}$ M compared with baseline; Fig. 3). At the maximum concentration of $10^{-8}$ M, S6c reduced diameter by $-76.2 \pm 0.7\%$ compared with baseline diameter ($P < 0.0001$). In the vessels from animals on a HS diet, the lowest concentration of S6c, $10^{-12}$ M, caused vasodilation; vessel diameter increased by $+14.4 \pm 7.1\%$ compared with baseline ($P = 0.03$). At the next higher concentration of $10^{-11}$ M, the afferent arteriolar response reverted to vasoconstriction, as evidenced by a return of afferent arteriolar diameter to control. Subsequently, higher concentrations of S6c caused further vasoconstriction, with a maximum reduction in diameter of $-62.4 \pm 7.1\%$ at a concentration of $10^{-8}$ M ($P < 0.001$). Thus S6c produced significantly less vasoconstriction in the HS vs. the NS group ($P = 0.0003$).

**Afferent arteriolar response to NE during NS and HS.** In the NS group, NE caused a concentration-dependent vasoconstriction (Fig. 4). The highest concentration of $10^{-6}$ M reduced diameter by $-71.8 \pm 7.9\%$ ($P < 0.001$). Similarly, in vessels from animals fed a HS diet, NE produced a similar concentration-dependent reduction in diameter that was no different
from responses observed in vessels from the NS group. The highest concentration of 10^{-6} M reduced diameter by -73.7 \pm 1.5\% compared with baseline (P < 0.001).

**Afferent arteriolar response to ET-1 during ET_B receptor blockade.** Blockade of ET_B receptors had no effect on afferent arteriolar diameters in vessels from rats on a NS diet (16.7 \pm 1.8 \mu m before vs. 16.5 \pm 1.6 \mu m during ET_B receptor blockade; NS). Similarly, ET_B receptor blockade had no effect on baseline diameter in vessels from rats on a HS diet (17.5 \pm 1.1 \mu m before vs. 17.2 \pm 1.2 \mu m during ET_B receptor blockade; NS). During ET_B receptor blockade, vasoconstriction to lower concentrations of ET-1 was abolished and higher concentrations of ET-1 were needed to induce a significant vasoconstriction in vessels from rats on both NS and HS diets (both P = 0.01 for 10^{-8} M compared with baseline; Fig. 5). There was no difference in the ET-1 response between the NS and the HS groups during ET_B receptor blockade.

**Afferent arteriolar response to ET-1 during ETA receptor blockade.** Blockade of ETA receptors had no effect on afferent arteriolar diameters in vessels from rats on a HS diet (14.5 \pm 0.9 \mu m before vs. 14.0 \pm 0.7 \mu m during ETA receptor blockade; NS). In the vessels from rats fed a HS diet, concentrations of ET-1 from 10^{-12} to 10^{-11} M led to significant vasodilation (Fig. 6; P = 0.02 and P = 0.04, respectively, compared with baseline). At a concentration of 10^{-10} M, the vascular response reverted to vasoconstriction (NS compared with baseline). The highest concentration of 10^{-8} M reduced diameter by -74.2 \pm 2.4\% compared with baseline diameter (P < 0.001). The initial vasodilatation in vessels from rats treated with a HS diet is in contrast to what we observed previously in vessels from rats on a NS diet, where an initial vasodilatory response was not detectable (23).

**Expression of ETA and ET_B receptors in renal microvessels during a NS and HS diet.** To determine whether changes in receptor expression could account for the functional differences we observed, preglomerular microvessels were isolated from animals on NS and HS diets. Expression of ETA receptors in preglomerular microvessels was not different between rats treated with a HS diet or a NS diet (41.0 \pm 4.8 vs. 32.4 \pm 9.9\% of \beta-actin control; NS) (Fig. 7). In contrast, expression of ET_B receptors was increased in microvessels from rats treated with a HS diet compared with vessels from rats on a NS diet (17.7 \pm 2.4 vs. 6.6 \pm 3.0\% of \beta-actin control; P = 0.02).

**DISCUSSION**

The main finding of the current study is that a HS diet leads to a rightward shift in the concentration-response relationship of endothelin peptides in afferent arterioles. In fact, an initial vasodilation was observed in arterioles from rats on a HS diet, but not a NS diet. These results are consistent with an enhancement of ET_B receptor-mediated, endothelium-dependent vasodilation during HS. ET_B receptor blockade, but not ETA receptor blockade, abrogated the vasodilatory response to ET-1.
during a HS diet. In line with these functional changes, we observed an upregulation of ETB receptor protein in renal microvessels during HS. These results provide strong evidence for increased endothelial ETB receptor function in the renal microcirculation of rats on a HS diet.

Because of the importance of afferent arteriolar tone for renal function and long-term blood pressure regulation, we examined the effects of a HS diet on the response of afferent arterioles to endothelin peptides. Renal vasoconstriction is a very powerful mechanism for inducing arterial hypertension (14). Renal vascular tone, as with any other vascular bed, depends on the balance between vasoconstrictor and vasodilatory factors. Maintaining this balance in afferent arterioles, in particular, during a HS diet, might be an important mechanism guarding against the development of arterial hypertension. An enhancement of endothelial ETB receptor-mediated afferent arteriolar vasodilation may serve to restore sodium balance by increasing renal blood flow and sodium excretion during a HS diet.

We have recently documented that ET-1 is a very potent constrictor of afferent arterioles (23). In fact, the renal vasculature seems to be more sensitive to the vasoconstrictor effects of ET-1 than the systemic circulation (34). A HS diet has been shown to increase vascular expression of ET-1, although this is not a universal finding (20, 26, 29). We have previously observed that rats fed a HS diet for 2 wk had an increase in urinary excretion of immunoreactive endothelin by >250% (36). Because urinary endothelin derives exclusively from the kidney (5), this is consistent with increased renal production of ET-1 during a HS intake. The exact intrarenal locations where enhanced ET-1 production occurs during a HS diet remain to be clarified. However, increased local production of ET-1 in afferent arterioles would lead to vasoconstriction, if responsiveness of these vessels during a HS diet would go unchanged. In the superior mesenteric artery, constrictor responses to ET-1 are even enhanced by a high-salt intake (4), which would lead to further increases in vascular resistance in the face of enhanced ET-1 generation.

In the current study, we also investigated the effect of big ET-1 on afferent arterioles. Big ET-1 is a 38-amino acid precursor peptide, locally converted by endothelin-converting enzymes (ECE) to ET-1 (21 amino acids). Our data demonstrate that big ET-1 has less vasoconstrictor activity than equimolar concentrations of ET-1 in afferent arterioles. This may help to explain previously published in vivo data showing that a bolus injection of big ET-1 into Sprague-Dawley rats leads to a natriuretic response compared with an antinatriuretic response of a similar concentration of ET-1 (19). Local vascular ECE activity might be a rate-limiting factor for the vasoconstrictor action of big ET-1, but our study was not specifically designed to examine this in detail. The current results are also consistent with previous work suggesting that the renal circulation has relatively less ECE activity compared with the extrarenal circulation (32).

In afferent arterioles from rats on a HS diet, lower concentrations of big ET-1 led to vasodilation as opposed to vasoconstriction seen in kidneys from rats on a NS diet. With ET-1, an initial vasodilatory response was observed under HS conditions, whereas ET-1 caused strong vasoconstriction at concentrations of 10^{-11} M and above in vessels from rats on a NS diet. Because vasodilation to big ET-1 and ET-1 can only be mediated by the endothelial ETB receptor, we further substantiated that observation by application of the ETB receptor-specific agonist S6c. S6c induced an initial vasodilation instead of a vasoconstrictor response in vessels from rats fed a HS diet. Blockade of ETB receptors, but not ETA receptors, abrogated the initial vasodilation to ET-1 during HS, confirming that enhanced ETB receptor function is responsible for this effect. Because only ETB receptors on endothelial cells have vasodilatory actions, these results demonstrate that endothelial ETB

Fig. 7. Expression of ETα and ETβ receptors in preglomerular microvessels during NS (n = 4) and HS diets (n = 6) determined by densitometry (top) with respective immunoblots (bottom).
receptor function is enhanced during a HS diet. In addition to the initial vasodilation observed in response to big ET-1, ET-1, and S6c, vasoconstrictor responses at higher doses were also attenuated in the arterioles from rats on a HS diet. This can be due either to pronounced buffering of smooth muscle ET_A/ET_B receptor-mediated vasoconstriction by enhanced endothelial ET_B receptor-mediated vasodilation, a reduction of smooth muscle ET_A/ET_B receptor-mediated vasoconstriction, or a combination of both. The fact that during ET_B receptor blockade with 30 nM A-192621, which blocks both endothelial and smooth muscle ET_B receptors, vasoconstriction to higher levels of endothelin peptides was similar between the NS and HS groups, indicates that ET_A receptor function is probably not affected by a HS diet. In addition, ET_A receptor expression was similar in vessels from rats on a NS and a HS diet.

ET_B receptor expression was found to be upregulated in renal microvessels from rats on a HS diet. This suggests that increased endothelial ET_B receptor protein expression may account for the enhanced functional response of endothelial ET_B receptor activation in vessels from rats on a HS diet. However, our vessel preparation does not permit localization of the increased ET_B receptor expression, but the functional data suggests that this occurs in the endothelium. Future studies in mice with specific knockout of endothelial ET_B receptors may be able to provide a definitive answer to this question. It should be noted that in terms of regulation of ET_B receptors, mice overexpressing ET-1 in the endothelium also demonstrate increased expression of ET_B receptors (2). Other studies indicate, however, that the ET_B receptor is downregulated when exposed to increased concentrations of ET-1 (3, 35). Therefore, the signaling pathways regulating the expression of ET_B receptors under various conditions require further exploration.

To examine whether the rightward shifts in the response to endothelin peptides are an effect specific to the endothelin system, or a generalized attenuation of afferent arteriolar responsiveness to vasoconstrictors, we also examined the effect of a HS diet on the response to NE. No alteration in the responsiveness to NE was detected, indicating that a HS diet selectively affects the endothelin system.

When our results are compared with the available literature, it is important to note that most studies on the effects of a HS diet on the endothelin system have been performed using the DOCA-salt model. The mineralocorticoid hypertension of the DOCA-salt model is likely to influence vascular responses to endothelin peptides differently from a HS diet alone. In addition to the hypertension, endothelial dysfunction has been documented as a hallmark feature of the DOCA-salt model (16), but it is not present in animals treated with a HS diet only (41). Furthermore, data generated in DOCA-salt rats appear to be contradictory. For example, isolated, perfused kidneys of DOCA-salt rats respond to an ET_B receptor agonist with vasodilation and loss of nitric oxide compared with vasodilation in normotensive control rats (18), indicating reduced function of the endothelial ET_B receptor. In whole-animal studies, however, we and others observed a more pronounced increase in blood pressure during ET_B receptor blockade in DOCA-salt rats compared with control rats (15, 31). This would indicate enhanced endothelial ET_B receptor function. Published data on the response of mesenteric arteries to ET-1 in the DOCA-salt model are also conflicting. Some authors report enhanced vasoconstrictor responses to ET-1 during DOCA-salt treatment (7), and others observe an attenuated response (8, 28). Similarly, in coronary vessels, some groups have found endothelin-induced contractions to be reduced in the DOCA-salt model (13), while others found the opposite (27). Although we lack any obvious explanation for these discrepancies, use of different rat strains may partly account for the observed differential responses.

Few studies have investigated the effect of a HS diet only, without DOCA application. Ballew et al. (4) showed an increased response to ET-1 in superior mesenteric arteries in rats treated with HS only. This is in contrast to our data, but the effects of a HS diet on vascular responses to endothelin peptides might also differ between vascular beds. Our results of enhanced ET_B receptor function, however, are in accordance with data from Giardina et al. (12), who found enhanced endothelium-dependent vasodilation via ET_B receptors in aortas of Sprague-Dawley rats on a HS diet.

In a previous report, we noted that ET_B receptor-mediated increases in renal medullary blood flow are enhanced during a HS diet (38). Our current results indicate that vasoconstrictor responses to endothelin peptides are attenuated in afferent arterioles from rats on a HS diet and even turned into vasodilatory responses at lower peptide levels, mediated by increased endothelial ET_B receptor function. Taken together, these data suggest that enhanced renal ET_B receptor function may be an important antihypertensive mechanism during a HS diet, restoring sodium balance by vasodilatory effects on the afferent arteriole and by increasing medullary blood flow. In line with this notion, pharmacological blockade of ET_B receptors or disruption of the ET_B receptor gene has been shown to produce salt-sensitive hypertension (11, 33). Thus it should be determined in future studies whether ET_B receptor function is impaired in salt-sensitive models of hypertension, such as in the DOCA model or in the Dahl salt-sensitive rat.

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