Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles

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Submitted 8 October 2002; accepted in final form 2 April 2003

Johnson, Fruzsina K., and Robert A. Johnson. Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. Am J Physiol Regul Integr Comp Physiol 285: R536–R541, 2003. First published April 3, 2003; 10.1152/ajpregu.00624.2002.—Vascular tissues express heme oxygenase, which metabolizes heme to form carbon monoxide (CO). CO promotes relaxation of vascular smooth muscle but also inhibits nitric oxide (NO) formation. This study examines the hypothesis that CO promotes endothelium- and NO synthase-dependent vasoconstriction of isolated arterioles. Studies were conducted on pressurized first-order gracilis muscle arterioles isolated from anesthetized male Sprague-Dawley rats. Exogenous CO, as well as a heme precursor, δ-aminolevulinic acid (δ-ALA), constricted arterioles with intact endothelium pretreated with phenylephrine; these effects were abolished by removal of the endothelium. CO- and δ-ALA-induced vasoconstrictions were converted to dilations by pretreatment with an inhibitor of NO synthase, Nω-nitro-l-arginine methyl ester, or with Nω-nitro-l-arginine methyl ester and an NO donor, sodium nitroprusside. Furthermore, CO-induced vasoconstriction was prevented by pretreatment with the NO synthase substrate I-arginine. This study shows that exogenous, as well as endogenously formed, CO can promote endothelium-dependent vasoconstriction in isolated gracilis muscle arterioles. Because CO-induced vasoconstriction is abolished by NO synthase blockade and by I-arginine, CO most likely promotes endothelium-dependent vasoconstriction by inhibiting endothelial NO formation.

METHODS

Chemicals

CO gas was purchased from Air Liquide (Harvey, LA), δ-aminolevulinic acid (δ-ALA) from Frontier Scientific (Logan, UT), Nω-nitro-l-arginine methyl ester (l-NAME), l-arginine hydrochloride, sodium nitroprusside (SNP), phenylephrine, and acetylcholine from Sigma Aldrich (St. Louis, MO), and all other chemicals from Fisher Scientific (Houston, TX). l-NAME, SNP, phenylephrine, l-arginine, and acetylcholine were dissolved in modified Krebs buffer immediately before use. CO-saturated solution (1 mmol/l) was prepared by bubbling ice-cold modified Krebs buffer with CO gas for 20 min. The final concentration of CO in the microvessel chamber (0.1–100 μmol/l) was achieved by predilution with ice-cold modified Krebs buffer in the infusion syringe (for 0.1, 1, and 10 μmol/l) and final dilution with superfusion buffer (modified Krebs buffer at 37°C). Stock and final microves sel chamber concentrations of CO were confirmed by combining samples with freshly lysed rat blood and then calculating from the resulting carboxyhemoglobin content (model OSM3, Radiometer, Westlake, OH). The composition of modified Krebs buffer was (mmol/l) 118.5 NaCl, 4.7 KCl, 1.4 CaCl2, 1.2 KH2PO4, 1.1 MgSO4, 25.0 NaHCO3, and 11.1 dextrose.

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Animals

Use of male Sprague-Dawley rats (250–300 g, n = 70; Harlan, Indianapolis, IN) in these studies was approved by the Institutional Animal Care and Use Committee. Rats were housed in a controlled environment and had free access to commercial rat chow and tap water.

Microvascular Preparation

Rats were anesthetized with a single dose of thiobutabarbital sodium (Inactin, 140 mg/kg ip) and heparinized (1,000 U/kg iv). The gracilis anticus muscles were removed, and segments of the first-order gracilis muscle arterioles were isolated as previously described (19). Individual arteriolar segments were cannulated at both ends with glass micropipettes in a water-jacketed vessel chamber (18 ml volume; Instrument Shop, New York Medical College, Valhalla, NY). Silicone tubing (Masterflex, Cole-Parmer, Vernon Hills, IL) connected the distal micropipette to a stopcock and the proximal micropipette to a reservoir containing modified Krebs buffer. The height of the reservoir was adjusted to 108.8 cm above the level of the arteriole to achieve 80 mmHg intraluminal pressure. The vessel chamber was superfused continuously via a nonrecirculating system (Masterflex L/S pump, Cole-Parmer) with oxygenated (14% O2-5% CO2-balance N2) modified Krebs buffer (5 ml/min) at 37°C (Isotemp 2100 immersion circulator, Fisher Scientific). For internal diameter measurements, the vessel chamber was mounted on the stage of a microscope (Micromaster, Fisher Scientific) that was fitted with a video camera (video microscope package, Fisher Scientific) leading to a video caliper (Texas A & M University, College Station, TX) and a television-videocassette recorder (Zenith, Sears). With this setup, a magnified image of the arteriolar segment was viewed on the television screen, and internal diameter was measured throughout the experiment by manual adjustment of the white guides superimposed by the video caliper.

Mounted vessels were allowed to stabilize for 60 min before initiation of the experiments. Only vessels that developed an active tone during the stabilization period were used for the studies. Pretreatment drugs (i.e., phenylephrine, l-NAME, SNP, or L-arginine) were included in the superfusion buffer. CO-saturated solution (1 mmol/l) or δ-ALA stock solution (80 mmol/l) was added directly to the microvessel chamber and then continuously infused (model M361, Sage, Boston, MA) into the superfusion buffer to quickly achieve and maintain the desired concentration of CO (0.1–100 μmol/l) or δ-ALA (80 μmol/l) in the chamber without interruption of chamber superfusion.

Experimental Design

Arterioles with intact endothelium. The following experiments were designed to study endothelium-dependent effects of exogenous and endogenously formed CO on arteriolar diameter. The arteriolar endothelium was left intact, and microvessels were pretreated with a vasoconstrictor, phenylephrine (100 mmol/l); phenylephrine treatment was continued throughout the remainder of the experiment. This concentration of phenylephrine (100 mmol/l) was used to match the internal diameter of denuded and l-NAME-pretreated vessels. After 25 min of pretreatment, CO (0.1–100 μmol/l) or δ-ALA (80 μmol/l) was administered, and internal diameter was monitored for 20 min. Recently, it has been reported that small amounts of CO release NO (0.01 μmol CO/l) and promote vasodilation (0.1 μmol CO/l), but physiological concentrations of CO (1–10 μmol/l) inhibit NO synthesis and release in isolated renal resistance vessels (21). To explore this potentially bimodal response, we examined the effects of exogenous CO at 0.1–100 μmol/l in arterioles with intact endothelium. Endogenous free heme levels are estimated to be ~0.5 μmol/l (6). Eight δ-ALA molecules are used for the synthesis of one heme molecule, and we used 80 μmol/l δ-ALA to enhance heme synthesis. At the end of the experiment, the presence of functional endothelium was confirmed by studying the vasodilatory responses to acetylcholine (1 μmol/l).

Arterioles denuded of endothelium. The following experiments were designed to study the endothelium-independent effects of exogenous and endogenously formed CO on arteriole diameter. After the stabilization period, the arteriolar endothelium was removed by perfusion of 2 ml of air through the lumen, as previously described (19). After an additional 40-min stabilization period, CO (50 μmol/l) or δ-ALA (80 μmol/l) was administered, and internal diameter was monitored for 20 min. At the end of the experiments, the absence of functional endothelium was confirmed by the lack of vasodilatory response to acetylcholine (1 μmol/l).

Arterioles pretreated with l-arginine. The following experiments were designed to study the endothelium-independent effects of exogenous CO on arteriole diameter. The microvessel endothelium was left intact. After the stabilization period, microvessels were exposed to an inhibitor of NO synthase, L-NAME (1 mmol/l). Exposure to the inhibitor was continued throughout the remainder of the experiment. Previous experiments have found that this concentration of l-NAME was required to cause maximal constriction of isolated gracilis muscle arterioles (unpublished observations), suggesting maximal achievable blockade of the NO system. After 55 min of pretreatment, CO (50 μmol/l) or δ-ALA (80 μmol/l) was administered, and internal diameter was monitored for 20 min.

Arterioles with “NO clamp.” The following experiments were designed to identify the NO synthase-independent effects of exogenous CO on arteriolar diameter. The microvessel endothelium was left intact. After the stabilization period, arterioles were pretreated with l-NAME (1 mmol/l) to block NO synthase activity and with an NO donor, SNP (10–30 mmol/l), to maintain internal diameter at before-pretreatment levels (NO clamp). Exposure to the clamp was continued throughout the remainder of the experiment. With this design, we achieved maximal blockade of NO synthase while maintaining the activity of the vascular NO signaling system by replacing endogenous NO with an NO donor. After a 55-min pretreatment period, CO (50 μmol/l) was administered, and internal diameter was monitored for 20 min.

Arterioles pretreated with l-arginine. The following experiments were designed to study the l-arginine-dependent effects of exogenous CO on arteriolar diameter. The microvessel endothelium was left intact. After the stabilization period, arterioles were pretreated with a vasoconstrictor, phenylephrine (100 mmol/l), and the substrate for NO synthesis, L-arginine (1 mmol/l). This pretreatment regimen was continued throughout the remainder of the experiment. With this design, we provided excess substrate for NO synthesis. After 60 min of pretreatment, CO (50 μmol/l) was administered, and internal diameter was monitored for 20 min.

Statistics

Values are means ± SE. Data showing time-related vascular responses were analyzed by analysis of variance, and further post hoc comparisons were made by performing orthogonal contrasts (18) using a statistical package (SYSTAT)
when appropriate. Data showing concentration-related vascular responses to exogenous CO were analyzed by t-tests. $P < 0.05$ was considered statistically significant.

RESULTS

During the stabilization period, internal diameter of isolated gracilis muscle arterioles decreased spontaneously (from 195 ± 2 to 131 ± 3 μm, $n = 70$, $P < 0.05$). Phenylephrine (100 nmol/l) pretreatment promoted concentration-dependent vasoconstriction (from 132 ± 3 to 98 ± 3 μm, $n = 35$, $P < 0.05$). Removal of the endothelium resulted in a decrease in internal diameter (from 144 ± 10 to 110 ± 6 μm, $n = 12$, $P < 0.05$). Pretreatment with an inhibitor of NO synthase, L-NAME (1 mmol/l), resulted in a comparable vasoconstriction (from 123 ± 7 to 85 ± 9 μm, $n = 10$, $P < 0.05$). However, the NO clamp, i.e., pretreatment with an NO synthase inhibitor, L-NAME (1 mmol/l), and an NO donor, SNP (10–30 nmol/l), caused a moderate increase in arteriolar diameter (from 101 ± 12 to 115 ± 10 μm, $n = 6$, $P < 0.05$). Internal diameter was larger in arterioles pretreated with phenylephrine (100 nmol/l) and L-arginine (1 mmol/l) than in vessels pretreated with phenylephrine only (99 ± 6 μm ($n = 7$) vs. 91 ± 5 μm ($n = 14$, $P < 0.05$).

In arterioles with intact endothelium (Fig. 1A), exogenous CO (50 μmol/l) promoted a sustained vasoconstriction (from 85 ± 5 to 66 ± 4 μm, $n = 8$, $P < 0.05$). Removal of the endothelium (Fig. 1B) abolished the vasoconstrictor response to CO (from 117 ± 9 to 117 ± 9 μm, $n = 8$, $P > 0.05$). Figure 2 shows concentration-dependent responses to exogenous CO in arterioles with intact endothelium measured as the average of the last four measurements (1 measurement/min) at the end of the 20-min exposure. Figure 2 shows that CO promoted concentration-dependent vasoconstriction that was evident at 1 μmol/l ($\Delta_{\text{max}} = -6 ± 1$ μm, $n = 5$, $P < 0.05$ vs. vehicle) and maximum at 50 μmol/l ($\Delta_{\text{max}} = -17 ± 3$ μm, $n = 8$, $P < 0.05$ vs. vehicle). In arterioles with intact endothelium (Fig. 3A), pretreatment with an NO synthase inhibitor, L-NAME (1 mmol/l), converted CO-induced vasoconstriction to vasodilation (from 91 ± 14 to 128 ± 11 μm, $n = 6$, $P < 0.05$). Similarly, in arterioles subjected to NO clamp (Fig. 3B), i.e., pretreated with an NO synthase inhibitor (1 mmol l-NAME/l) and an NO donor (10–30 nmol SNP/l), CO caused vasodilation (from 115 ± 10 to 123 ± 9 μm, $n = 6$, $P < 0.05$). The heme synthesis precursor δ-ALA (80 μmol/l) promoted vasoconstriction in arterioles with intact endothelium ($\Delta_{\text{max}} = -9 ± 1$ μm, $n = 6$, $P < 0.05$; Fig. 4). δ-ALA-induced vasoconstriction was converted to vasodilation by removal of

![Figure 1. Effects of exogenous carbon monoxide (CO, 50 μmol/l) on internal diameter of isolated pressurized 1st-order gracilis muscle arterioles in the presence of endothelium pretreated with phenylephrine (100 nmol/l; A) and in the absence of functional endothelium (B). Values are means ± SE. *$P < 0.05$ relative to control (before CO treatment).](image1)

![Figure 2. Effects of exogenous CO concentration ([CO], 0.1–100 μmol/l) or matched iced-cold buffer vehicle (Veh) on internal diameter of isolated pressurized 1st-order gracilis muscle arterioles in the presence of endothelium pretreated with phenylephrine (100 nmol/l). Maximal response was calculated as average of the last 4 measurements (1 measurement/min) at the end of the 20-min exposure period. Values are means ± SE; $n = 5$ for vehicle, $n = 4$ for 0.1 μmol/l CO, $n = 5$ for 1 μmol/l CO, $n = 4$ for 10 μmol/l CO, $n = 8$ for 50 μmol/l CO, and $n = 3$ for 100 μmol/l CO. *$P < 0.05$ vs. Veh.](image2)

![Figure 3. Effects of exogenous CO (50 μmol/l) on internal diameter of isolated pressurized 1st-order gracilis muscle arterioles in the presence of functional endothelium pretreated with an NO synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 1 mmol/l; A) and pretreated with L-NAME (1 mmol/l) and an NO donor, sodium nitroprusside (10–30 nmol/l; B). Values are means ± SE. *$P < 0.05$ relative to diameter before CO treatment.](image3)
the endothelium ($\Delta_{\text{max}} = 7 \pm 1 \, \mu\text{m}, n = 4, P < 0.05$) and pretreatment with an inhibitor of NO synthase, L-NAME (1 mmol/l; $\Delta_{\text{max}} = 7 \pm 1 \, \mu\text{m}, n = 4, P < 0.05$). In arterioles with intact endothelium (Fig. 5B), pretreatment with the NO synthase substrate L-arginine (1 mmol/l) prevented CO-induced vasoconstriction (from 100 ± 6 to 95 ± 6 $\mu$m, $n = 7, P > 0.05$).

**DISCUSSION**

The present study shows that CO and a heme precursor, which drives endogenous CO formation, can promote vasoconstriction in a manner that is endothelium dependent and L-NAME sensitive.

CO is commonly characterized as a vasodilatory regulator of vascular tone, inasmuch as CO has been shown to relax vascular smooth muscle (2, 3, 6, 22, 24). In addition, numerous studies have reported that exogenously applied and endogenously formed CO promoted dilation of different vascular beds (2, 3, 6, 22, 24). With regard to these findings, we have similarly observed that heme-derived CO dilated isolated gracilis muscle arterioles pretreated with an inhibitor of NO synthase (10). However, a commonly overlooked effect of CO is inhibition of NO synthesis by binding to the NO synthase (1, 9, 14, 25), a recent report suggested that endogenous CO may also promote hypertension by serving as a partial agonist/antagonist for soluble guanylate cyclase (4). In that case, in arterioles pretreated simultaneously with an NO synthase inhibitor and an NO donor (i.e., NO clamp), CO should promote vasoconstriction. However, our present study shows that exogenous CO promotes concentration-dependent vasoconstriction in isolated rat arterioles with intact endothelium. Because the vasoconstrictive effect of CO was abolished by removal of the endothelium, we reasoned that CO might (1) stimulate release of an endothelium-derived constricting factor or (2) inhibit release of an endothelium-derived relaxing factor. Because previous studies suggested that CO interferes with NO synthesis (1, 9, 14, 25), we examined the effects of exogenous CO in arterioles with intact endothelium pretreated with an inhibitor of NO synthase. Consistent with our previous reports (10), we have found that pretreatment with L-NAME converted CO-induced vasoconstriction to vasodilation. These data suggest that CO-induced endothelium-dependent vasoconstriction is most likely due to interference with the vasodilatory effects of the endothelial NO system.

Although previous studies have shown that CO directly inhibits NO synthase (1, 9, 14, 25), a recent report suggested that endogenous CO may also promote hypertension by serving as a partial agonist/antagonist for soluble guanylate cyclase (4). In that case, in arterioles pretreated simultaneously with an NO synthase inhibitor and an NO donor (i.e., NO clamp), CO should promote vasoconstriction. However, we found that CO promotes vasodilation in vessels subjected to the NO clamp. Nevertheless, the response was marginally attenuated compared with arterioles pretreated only with the NO synthase inhibitor. Although this attenuation may in part be due to CO interfering with NO-induced vasodilatory mechanisms, we did not find vasoconstriction in these arterioles subjected to NO clamp; this feature strongly suggests that CO-induced vasoconstriction does not arise primarily from inhibition of soluble guanylate cyclase activity.

Our present study shows that exogenous CO promotes concentration-dependent vasoconstriction in isolated rat arterioles with intact endothelium. Because the vasoconstrictive effect of CO was abolished by removal of the endothelium, we reasoned that CO might (1) stimulate release of an endothelium-derived constricting factor or (2) inhibit release of an endothelium-derived relaxing factor. Because previous studies suggested that CO interferes with NO synthesis (1, 9, 14, 25), we examined the effects of exogenous CO in arterioles with intact endothelium pretreated with an inhibitor of NO synthase. Consistent with our previous reports (10), we have found that pretreatment with L-NAME converted CO-induced vasoconstriction to vasodilation. These data suggest that CO-induced endothelium-dependent vasoconstriction is most likely due to interference with the vasodilatory effects of the endothelial NO system.

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**Fig. 4.** Effects of a heme synthesis precursor, δ-aminolevulinic acid (δ-ALA, 80 μmol/l) on internal diameter of isolated pressurized 1st-order gracilis muscle arterioles in the absence of functional endothelium, in the presence of endothelium pretreated with phenylephrine (100 nmol/l), and in the presence of endothelium pretreated with an inhibitor of NO synthase, L-NAME (1 mmol/l). Values are means ± SE. *P < 0.05 relative to control (before δ-ALA treatment) in endothelium-denuded and phenylephrine-pretreated vessels. †P < 0.05 relative to control (before δ-ALA treatment) in L-NAME-pretreated vessels.

**Fig. 5.** Effects of exogenous CO (50 μmol/l) on internal diameter of isolated pressurized 1st-order gracilis muscle arterioles in the presence of endothelium pretreated with phenylephrine (100 nmol/l; A) and pretreated with phenylephrine (100 nmol/l) and the NO synthase substrate L-arginine (1 mmol/l; B). Values are means ± SE. *P < 0.05 relative to control (before CO treatment).
The major source of endogenous CO production is the enzymatic degradation of heme by heme oxygenase (20). δ-ALA is a precursor for heme synthesis: eight δ-ALA molecules are required for the synthesis of one molecule of heme (15, 17). In the heme synthetic cascade, δ-ALA formation is the primary point of regulation (15, 17). Provision of exogenous δ-ALA drives heme synthesis (11) and, consequently, increases substrate availability for heme oxygenase. Radiolabeled δ-ALA has been shown to generate radiolabeled CO in a reaction that is mediated by heme oxygenase (11). Because heme oxygenase activity appears to be regulated by substrate availability (6, 16), provision of δ-ALA increases heme oxygenase activity and, consequently, endogenous CO formation (12, 26) in a concentration-dependent manner (12), and the iron released during heme degradation is available to be recycled into the formation of new monomeric heme (17). Our previous studies (6, 10) used heme preparations to provide substrate for heme oxygenase (6, 16). However, heme contains iron, and its use may potentially contribute to detrimental iron loading of the tissues. To minimize the chances of these unwanted effects, we decided to use the heme synthesis precursor δ-ALA to drive heme synthesis and, consequently, drive the endogenous formation of CO. In the present study, we observed that the δ-ALA promoted effects that were similar to those of exogenous CO. In arterioles with intact endothelium, δ-ALA promoted vasoconstriction, and this vasoconstrictive effect was converted to vasodilation by removal of the endothelium or pretreatment with an inhibitor of NO synthase. The highly unique actions of this well-established heme precursor, in conjunction with the highly unique actions of exogenously formed CO, strongly suggest that δ-ALA drives CO formation in these isolated microvessels and that its vasoconstrictive actions arise from the subsequent generation of CO by inhibiting the vasodilatory effects of the NO system.

A previous study using isolated NO synthase suggested that excess substrate (L-arginine) levels decrease the affinity of CO binding to NO synthase (1, 14) by binding close to the heme and/or altering the confirmation of the distal heme pocket and, hence, constraining CO binding to the heme moiety (1, 14). To investigate whether L-arginine protects against CO-induced vasoconstriction, we conducted experiments on arterioles with intact endothelium pretreated with L-arginine. We found that, in isolated gracilis muscle arterioles, pretreatment with L-arginine (1 mmol/l) abolished CO-induced vasoconstriction. These data show that L-arginine can protect against CO-induced vasoconstriction, which further supports the notion that CO might promote vasoconstriction by inhibiting endothelial NO synthase.

The vasodilatory effects of exogenous CO and δ-ALA in arterioles pretreated with L-NAME appear to reflect their actions on vascular smooth muscle. Our findings show that the vasodilatory effects of CO may dominate in the absence of NO synthase activity when the endothelium is present. However, our findings also show that exogenous CO, at the examined concentration, has little vasodilatory action in the absence of endothelium. Because L-NAME-pretreated and endothelium-denuded vessels should lack NO synthase activity, the difference in CO-induced responses suggests that perhaps other endothelium-dependent systems may be involved. NO has been suggested to interact with other vasoactive mediators produced in the vascular endothelium, such as endothelin and prostaglandins. Many of these systems may be altered when NO synthesis is blocked. CO has also been suggested to interact with some of these other endothelium-dependent vasoactive mediators (6). In the face of these observations, the possibility exists that, in L-NAME-pretreated vessels, increased release of an endothelium-dependent vasoconstrictor or decreased release of an endothelium-dependent vasodilator may contribute to the vasodilatory effects of CO. However, exploration of such potential interactions is beyond the scope of the present study.

A recent study (21) reported that small amounts of CO release NO (0.01 μmol CO/l) and promote vasodilation (0.1 μmol CO/l), but physiological concentrations of CO (1–10 μmol/l) inhibit NO synthesis and release in isolated renal resistance vessels. In our preparation, we found that CO at 0.1 μmol/l did not produce statistically significant changes in arteriolar diameter compared with the matched unblocked ice-cold vehicle controls. At larger concentrations, exogenous CO promoted concentration-dependent vasoconstriction of arterioles with intact endothelium that was evident at 1 μmol/l and maximal at 50 μmol/l. Because tissue contents of CO were reported to be 1–50 pmol/mg fresh weight (23), which roughly translates to 1–50 μmol/l, it appears that CO can affect NO synthase activity at “physiological” concentrations.

It also appears that the inhibitory effect of CO on endothelial NO synthase may become more pronounced during certain pathophysiological conditions when heme oxygenase expression and/or endogenous CO formation is increased. Specifically, induction of heme oxygenase-1 has been shown to attenuate muscarinic agonist-induced NO release (21) and vasodilation (7) in isolated renal resistance vessels. We recently reported that vascular heme oxygenase-1 levels were increased in Dahl-Rapp salt-sensitive rats with salt-induced hypertension (5). Skeletal muscle arterioles isolated from these hypertensive rats showed abolished endothelium-dependent vasodilatory responses, which were completely restored by acute in vitro treatment with an inhibitor of endogenous CO formation (5). Therefore, it appears that endogenous levels of CO are able to inhibit endothelial NO synthase activity under physiological conditions, but this inhibitory effect becomes more severe during certain pathophysiological conditions when endogenous CO formation is increased.

In summary, our data show that, in isolated gracilis muscle arterioles with intact endothelium, CO and a heme synthesis precursor, δ-ALA, promote endothelium-dependent vasoconstriction. In arterioles pre-
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