Continuous Perivascular L-Arginine Delivery Increases Total Vessel Area and Reduces Neointimal Thickening After Experimental Balloon Dilatation

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Abstract—The aim of this study was to evaluate whether vascular remodeling and neointimal thickening occur after balloon dilatation of the nonatherosclerotic rabbit carotid artery, and whether both processes are influenced by continuous perivascular delivery of L-arginine or the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). In the first experiment, histological and morphometric evaluation of arteries was performed at different time points after balloon dilatation: 10 minutes (n=7), and 1 (n=7), 2 (n=9), 3 (n=20), or 10 (n=5) weeks. Neointimal thickening progressively contributed to luminal narrowing for at least 10 weeks after angioplasty. During the first 2 weeks after dilatation, a significant decrease of the total vessel area was measured. Ten weeks after dilatation, both the neointimal and total vessel area were increased without further changing of the luminal area. In the second experiment, endothelial injured rabbits were randomly assigned to receive 2 weeks of continuous local perivascular physiological salt solution (n=6), L-arginine (n=8), or L-NAME (n=7), starting immediately after balloon dilatation (ie, local drug delivery during the first phase of the biphasic vascular remodeling process). Perivascular L-arginine delivery significantly reduced the neointimal area, despite an increased number of neointimal Ki-67-positive smooth muscle cells. Both the luminal area and total vessel area were significantly increased. Serum L-arginine levels remained unchanged. L-NAME administration had no effect on the neointimal area, nor on the luminal and total vessel area. Neointimal formation and biphasic vascular remodeling occur after experimental balloon dilatation of the nonatherosclerotic rabbit carotid artery, and can be influenced by continuous local perivascular delivery of L-arginine.

Key Words: balloon dilatation • neointima • remodeling • restenosis • nitric oxide • L-arginine

Restenosis remains a major limitation of the long-term success of percutaneous transluminal coronary angioplasty (PTCA). Although the mechanisms responsible for this process are not completely understood, neointimal thickening certainly contributes to it. Recent findings have suggested that restenosis may also be related to reactive and adaptive remodeling processes, probably initiated in the arterial adventitia. Glagov et al observed that the left main coronary artery in humans showed an adaptive vessel enlargement in response to progressive plaque expansion. Several intravascular ultrasound studies have shown that compensatory enlargement occurs throughout the coronary tree and preserves or augments lumen area during the early stages of atherosclerosis. In contrast, there is ample evidence that the reverse of this phenomena, reduction in total vessel area and lumen area, may occur after balloon angioplasty. Experimental studies recently suggested that this reduction of the total vessel area and lumen area is even more important than neointimal thickening in determining the final chronic lumen size after balloon angioplasty.

Intimal thickening and vascular remodeling are regulated by various substances, derived from endothelial cells, smooth muscle cells, platelets, macrophages, and leukocytes. One of these factors could be nitric oxide (NO), one of the most vasoactive autacoids. NO is important in regulating vascular tone, inhibits platelet aggregation, and limits platelet-endothelial interactions. Moreover, NO may play a role in maintaining the normal mitogenic state of vascular smooth muscle by inhibiting proliferation. Therefore, vascular NO is supposed to exert an inhibitory effect on some key processes in atherogenesis. In several experimental models of balloon dilatation, induction of nitric oxide synthase (iNOS) occurs within hours after injury and persists until weeks after dilatation. This iNOS is most likely expressed in neointimal smooth muscle cells or macrophages. Also, iNOS positive macrophages are described in the adventitia. The induction of nitric oxide synthase (NOS) after arterial injury by angioplasty may have some endogenous “antirestenotic” effect, and may partly explain why
long-term oral supplementation with L-arginine, the substrate of the enzyme, reduces intimal hyperplasia in these experimental models.31–33

The aim of our study was to evaluate whether vascular remodeling and neointimal thickening occur after experimental balloon dilatation in the nonatherosclerotic rabbit carotid artery, and whether one of these processes can be influenced by continuous local perivascular delivery of L-arginine or the NOS inhibitor N\(^5\)-nitro-L-arginine methyl ester (L-NAME).

Methods

Experimental Protocol

**Balloon Dilatation**

Male New Zealand White rabbits (2.5 to 3 kg; n=69), fed a normal laboratory diet without cholesterol supplementation, were anesthetized with sodium pentobarbital (30 mg/kg body weight, IV). The rabbits were posed in a supine position and the ventral neck region was infiltrated with xylocaine for local anesthesia. Both carotid arteries were surgically exposed and dissected from the surrounding tissues. For balloon dilatation, direct arteriotomy was performed in the right common carotid artery, just proximal of the bifurcation. A standard 20-mm-long, 2.5-mm PTCA balloon dilatation catheter (Advanced Cardiovascular Systems) was introduced over a 0.014-inch floppy guide wire and advanced retrogradely into a nonmanipulated region of the common carotid artery about 5 cm proximally from the incision, in the direction of the aorta. The balloon was inflated 3 times with an inflation pressure of 6 atm, for periods of 2 minutes each. Between the subsequent inflations the balloon was left deflated in the artery for 1 minute. After removal of the catheter, the small distal incision was closed surgically, with restoration of arterial blood flow.

The rabbits were fed standard chow after the intervention and were housed in accordance with Animal Welfare Act specifications. All surgical procedures conformed to the guidelines detailed in the “Position of the American Heart Association on Research Animal Use.”

**Description of Experiments**

In the first experiment, histological and morphometric evaluation of the complete angioplastied region (20 mm) was performed at different time points after balloon dilatation: 10 minutes (n=7), and 1 (n=7), 2 (n=9), 3 (n=20), or 10 (n=5) weeks. At the end of the experiment, and to prevent thrombus formation, the blood of the rabbits was anticoagulated with heparin (bolus of 150 U/kg, IV, added immediately after anesthesia). Ten minutes later, the animals were killed by an overdosage of sodium pentobarbital. After fixation, both the angioplastied region in the right carotid artery and the equivalent normal control region in the left carotid artery were surgically removed.

In the second experiment, 21 rabbits were randomly assigned to receive continuous perivascular treatment with physiological salt solution (n=6), L-arginine (n=8; Sigma), or L-NAME (n=7; Sigma). Osmotic minipumps (Alzet model 2 ML2; ALZA) were filled with sterile 0.9% NaCl, or with a sterile solution of about 1.4 mol/L L-arginine or 0.93 mol/L L-NAME that had been dissolved in deionized water. The concentrations were adapted to the initial weight of each rabbit and the factory-determined mean pumping rate of 5.18±0.20 μL/h to deliver 10 mg/kg per day for 2 weeks, both for L-arginine and L-NAME. The pumps were warmed up until rabbit body temperature (38°C) and surgically implanted immediately after balloon dilatation, in a perivascular pouch just beside the angioplastied arterial region. In the

| TABLE 1. Table of Ki-67-Positive Smooth Muscle Cells after Balloon Dilatation |
|------------------|--------|--------|--------|--------|
| Ki-67 + SMC/area (mm²) |       |        |        |        |
| Neointima         | ...    | 120±38* | 187±47* | 221±41* | 207±25* |
| Media             | 2±1    | 14±6*   | 8±5*    | 6±4*    | 3±2     |

Numbers expressed as mean±SEM. SMC indicates smooth muscle cell.

*P<0.05, significantly different than 10 min after balloon angioplasty.

**Light Microscopy–Morphometry**

**Immunohistological Techniques**

After fixation, arterial segments were cut into 4-mm lengths. At least 4 segments per artery were paraffin embedded. Transversal sections were stained with hematoxylin and eosin. After selection of the sirius red hematoxylin-stained material, immunohistochemistry was carried out. The following monoclonal antibodies were used: alpha-smooth muscle cell actin (1/5000 dilution; Sigma), the proliferation marker Ki-67 (1/100 dilution; Immunotech), platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31; 1/10 dilution; Dako), and rabbit antimalphaphage (RAM 11, 1/100 dilution; Dako). The antibodies were detected by an indirect peroxidase antibody conjugate technique. The sections were preincubated with BSA to prevent nonspecific binding of the primary antibody. The monoclonal antibodies were diluted in PBS. After 3 washes with PBS, the sections were incubated with rabbit anti-mouse peroxidase (Jackson ImmunoResearch) for 45 minutes. For the demonstration of the complex, 3-amin-9-ethylcarbazole was used as a chromogen. The specificity of these antibodies has been demonstrated in previous studies.34–37

The total number of smooth muscle cell (SMC) nuclei in the media and the neointima, and the number of Ki-67 immunoreactive nuclei in the media and the neointima were counted using a projection microscope. The mean of at least 4 transverse segments per artery was calculated.

**Histomorphometric Techniques**

Digital planimetry of at least 4 transversal segments per artery was performed with a computer-assisted morphometric program (Osteo Metrics). The area of the lumen and the areas circumscribed by the internal elastic lamina (IEL area) and the external elastic lamina (EEL area) were estimated by computerized measurement. The area of the media and intima were calculated by subtraction: medial area equals EEL area minus IEL area; intimal area equals EEL area minus lumen area. To ensure homogeneity of data, the absolute arterial and luminal areas (A) were also calculated from the computerized measurements of the perimeter lengths (C) of the EEL and lumen, assuming that EEL and luminal perimeter described perfect circles (A=C²/4π). The mean of at least 4 tissue segments per artery was chosen to be the final result. This method has also been used, in part, by Gertz et al.16

**Data Analysis**

All data are expressed as mean±SEM. The number of arteries reported equals the number of rabbits used. A 5% level of significance was selected. To compare areas of lumen, neointima, media, and total vessel, the number of nuclei per section, and the percentage of Ki-67-positive nuclei of the neointima and media between dilated and control arteries, we used a paired t test. To compare areas of dilated arteries over time, a nonparametric analysis of variance
(Friedman) was performed. Correlations between pairs of factors were evaluated with Spearman’s rank correlation.

**Results**

**Time Course**

From an initial weight averaging 2.8±0.1 kg, all rabbits gained weight during the follow-up period. The weight at the time of death was on average 3.0±0.1 kg. None of the rabbits died during follow-up.

**Light Microscopic Analysis**

Unoperated control arteries demonstrated an intact CD31-positive endothelial cell layer, lying directly on the internal elastic membrane, at all time points. Subendothelial alpha-smooth muscle actin positive cells were absent. The

**Figure 1.** Time course of luminal, neointimal, medial, and total vessel (lumen + neointima + media) area, both in unoperated (control) and dilated arteries. In unoperated arteries, neither of these areas significantly changes over time. In contrast, in dilated arteries a significant decrease of the luminal area compared with immediate postdilatation result is noticed (#P<0.05). Total vessel area is significantly decrease, also compared with immediate postdilatation result, 2 and 3 weeks after dilatation (*P<0.05). Ten weeks after dilatation, both neointimal and total vessel areas are increased without influencing the luminal area.

**Figure 2.** Time course of number of medial smooth muscle cell (SMC) nuclei/mm², both in operated (control) and dilated arteries. In unoperated vessels, number of medial SMC nuclei/mm² does not change over time. In dilated arteries, significant increase of number of medial SMC nuclei/mm² is noticed 1 week after dilatation (*P<0.05, compared with time control).
media was intact, with few Ki-67-positive smooth muscle cells.

In some balloon-treated arteries (12/48; 25%), an occlusive thrombus was observed, most probably related to severe injury. These arteries (1 week, n=2; 2 weeks, n=3; 3 weeks, n=6; 10 weeks, n=1) were excluded from further light microscopic and morphometric analysis.

In nonoccluded arteries, 10 minutes after balloon dilatation, marked segmental loss of smooth muscle cells and circular media tears were always observed. In most of the arteries, red blood cells were observed in sites of severe, deep injury of the media, sometimes reaching the adventitia. The fragmented internal elastic membrane was partially covered by sparse islands of remaining CD31-positive endothelial cells. One week later, a thin circular rim of neointima was observed, consisting of alpha-smooth muscle actin positive smooth muscle cells.

From 2 weeks on, the media appeared to be nearly completely repaired. A prominent circular neointima was observed in all specimens, lined by a continuous layer of CD31-positive cuboidal endothelial cells. The number of Ki-67-positive smooth muscle cells in neointima and media is presented in Table 1.
**Morphometric Analysis**

The areas of lumen, media, and the total vessel of the unoperated control arteries (n=36) remained constant throughout the whole period of investigation (Figure 1).

Ten minutes after balloon dilatation, the luminal (P=0.001) and total vessel (P=0.001) areas were significantly increased, compared with the unoperated contralateral controls. In contrast, the area of the media was significantly (P=0.001) decreased, most likely because of mechanical compression related to the balloon dilatation (Figure 1).

In the first 2 weeks after dilatation, the luminal area decreased progressively and significantly (1st week, P=0.003; 2nd week, P=0.001; both compared with the immediate postdilatation result). The total vessel area remained constant in the first week but declined significantly (P=0.001) in the second week, both also compared with the immediate postdilatation result. A thin neointima was already present after 1 week (Figure 1). The area of the media (P=0.002), and the number of medial smooth muscle cells (P=0.001) were significantly increased 1 week after dilatation, compared with the immediate postdilatation result, but returned to the levels of the contralateral controls within 2 weeks (Figures 1 and 2).

From the second week after balloon treatment, the luminal area remained unchanged. In contrast, both the neointimal area (3rd week, P=0.001; 10th week, P<0.001; both compared with 1st week) and the total vessel area (3rd
Effect of L-arginine and L-NAME
From an initial weight averaging 2.8±0.1 kg, all groups of rabbits gained weight during the 2-week treatment period, and had similar body weights at the time of death (on average 2.9±0.1 kg). None of the rabbits died during treatment. In none of the groups was a significant residual volume measured in the osmotic minipumps.

Measurement of L-Arginine Plasma Levels
Mean plasma L-arginine levels of L-arginine-supplemented animals were not significantly increased during treatment (before treatment: 209.6±28.0 μmol/L; after 1 week: 174.4±51.7 μmol/L; after 2 weeks: 195.7±31.8 μmol/L).

Light Microscopic Analysis
The unoperated control vessels displayed normal morphology in the 3 treatment groups.

Both in the L-arginine and L-NAME-supplemented animals, an occlusive organized thrombus was observed in one dilated artery. These 2 animals were excluded from further evaluation (2/21; 10%).

In the nonoccluded balloon-treated arteries, there were no qualitative morphologic differences among the 3 groups after 2 weeks of supplementation. All vessels demonstrated a consistent circumferential neointima, with largely intact internal and external elastic laminae, and media. The neointima was characterized by alpha-smooth muscle actin-positive smooth muscle cells, surrounded by connective tissue, and lined by CD31-positive endothelial cells. In the L-arginine-supplemented animals however, the neointima appeared to be markedly smaller, although smooth muscle cells in mitosis were frequently seen. The total vessel area appeared to be larger, compared with vehicle or L-NAME-supplemented animals (Figure 4).

Morphometric Analysis
The morphometry of the nondilated arteries was not influenced by vehicle, L-arginine or L-NAME supplementation (Table 2).

In the vehicle-supplemented animals (n=6), the luminal area (P=0.002) and total vessel area (P=0.001) of balloon-treated arteries were significantly decreased, compared with unoperated vessels, and not significantly different from the 2-week values in the first experiment. A prominent neointima was present. The medial area equaled that of the unoperated vessels (Table 2).

In the L-arginine-supplemented animals (n=7), the luminal area (P<0.001) and total vessel area (P<0.001) of balloon-treated arteries, were significantly increased, compared with the balloon-treated arteries in vehicle-supplemented animals. The luminal area and total vessel area were not significantly different from those of the unoperated contralateral arteries. The neointimal area in the balloon-treated arteries was significantly reduced (P<0.001), as compared with the vehicle-treated animals. The area of the media remained unchanged (Table 2).

In the L-NAME-supplemented animals (n=6), in balloon-treated arteries, the area of the lumen, the neointima, the media, and the total vessel were not significantly different from those observed in the vehicle-supplemented animals after angioplasty (Table 2).

In the unoperated control arteries, a significant positive, linear correlation existed again between lumen area and...
Ki-67 Immunoreactivity in Neointima and Media

In balloon-treated arteries of L-NAME-supplemented animals (n=6), the number of neointimal and medial SMCs, just as the percentage Ki-67-positive cells in neointima and media, were not significantly different compared with balloon-treated arteries in vehicle-supplemented animals (Table 2).

**Discussion**

Neointimal Growth and Arterial Remodeling After Balloon Dilatation in the Nonatherosclerotic Rabbit Carotid Artery

The present study demonstrates that luminal narrowing of the normal rabbit carotid artery after balloon dilatation results from both neointimal proliferation and biphasic remodeling of the vessel wall. Neointimal thickness progressively increases till at least 10 weeks after angioplasty. The early decrease of the total vessel area is followed by a progressive increase of the total vessel area during the next weeks, which compensates completely for the further increase of neointimal formation (Figure 1).

Our findings confirm the importance of arterial remodeling in restenosis after balloon dilatation. However, the observation that remodeling after injury can be a dynamic process of early decrease followed by late increase of the total vessel area, has to our knowledge not yet been documented. This may partly explain the variable results between some experimental studies, most evaluating remodeling about 4 weeks after angioplasty in atherosclerotic arteries. However, the ballooning condition used in our model is so severe that the ensuing changes in the carotid artery might not closely mimic the ones obtained by other authors using milder operating conditions. The cellular mechanisms responsible for the remodeling process remain largely speculative. Because our experimental model reflects a model of severe vascular injury with circular media tears reaching the adventitia after balloon dilatation, a possible contributing role may be played by activated migratory-competent cells coming from the circulating blood. The positive correlation which exists in our data

| Table 2. Effect of Perivascular Vehicle, L-Arginine, or L-NAME Delivery on Morphometric Appearance after Balloon Dilatation |
|-----------------|-----------------|-----------------|-----------------|
|                  | Vehicle (n=6)   | L-Arginine (n=7) | L-NAME (n=6)    |
|                  | Control Balloon | Control Balloon | Control Balloon |
| Lumen area (mm²) | 0.77±0.06       | 0.48±0.03       | 0.78±0.06       | 0.88±0.06       | 0.79±0.08       | 0.52±0.05       |
| Neointima        |                 |                 |                 |
| Area (mm²)       | ...             | 0.15±0.01       | ...             | 0.05±0.008*     | ...             | 0.16±0.02       |
| SMC nuclei/section | ...        | 751±98            | ...     | 438±87*         | ...             | 695±89          |
| Ki67+SMC nuclei (%) | ...     | 4±1             | ...     | 17±3*           | ...             | 3±1             |
| Ki67+SMC/area (mm²) | ...   | 200±57           | ...     | 1489±279*       | ...             | 112±43          |
| Media            |                 |                 |                 |
| Area (mm²)       | 0.48±0.05       | 0.48±0.02       | 0.48±0.04       | 0.48±0.02       | 0.48±0.06       | 0.48±0.02       |
| SMC nuclei/section | 1050±62    | 1150±78         | 1078±77         | 1124±94         | 1069±84         | 1189±67         |
| Ki-67+SMC nuclei (%) | 0.3±0.1 | 0.4±0.1         | 0.3±0.1         | 0.4±0.1         | 0.2±0.1         | 0.4±0.1         |
| Ki-67+SMC/area (mm²) | 7±3     | 10±3           | 7±3             | 9±3             | 7±3            | 10±3            |
| Total vessel area (mm²) | 137±0.09 | 1.15±0.09 | 1.35±0.08 | 1.50±0.11* | 1.38±0.01 | 1.21±0.07 |

Numbers expressed as mean±SEM. SMC indicates smooth muscle cell. *P<0.05, significantly different than vehicle-supplemented, balloon-treated arteries.
smooth muscle cells, as well as in neointimal and adventitial macrophages.26,27,29,30

In our study, L-arginine was delivered perivascularly by osmotic minipumps. This technique assures a very constant local dosage of the amino acid, administered very closely to the adventitia, the area where vascular remodeling is likely initiated.

The complete suppression by L-arginine of the early balloon dilatation-induced reduction in total vessel area is a new finding. It was not seen in other studies where vessel and luminal areas were measured 4 weeks after angioplasty.33 The lack of effect in the latter study may be caused by the timing of the end point, because our results suggest that the normally occurring reduction of total vessel area predominates at earlier stages. Moreover, pharmacokinetic differences related to the oral33 or perivascular administration could contribute to the discrepancy as well. In our study, mean plasma L-arginine levels were not significantly increased during L-arginine treatment; this indeed suggests local perivascular delivery. The mechanisms responsible for this intriguing “positive” modulation of early remodeling in nonatherosclerotic vessels by continuous perivascular L-arginine delivery remain largely speculative. The ability of L-arginine to increase endothelium-dependent relaxations has been well documented,39–41 but does not necessarily prove that vascular NO production is raised by perivascular L-arginine.49 L-arginine may also be involved in adventitial collagen synthesis, perhaps partly by influencing iNOS containing adventitial macrophages.29,30 Part of the beneficial effects of L-arginine may be caused by other mechanisms that are not directly related to NO production.50

The reduction of intimal thickening by local perivascular delivery of L-arginine is in accordance with observations that long-term oral supplementation with L-arginine reduces intimal hyperplasia after angioplasty.31–33 The inhibition of intimal thickening was to a large extent explained by the reduced accumulation of subendothelial smooth muscle cells in the intima of L-arginine-treated rabbits. This could be because of inhibition of smooth muscle cell migration from media to intima,42 inhibition of cell proliferation,43 an increase in apoptotic cell death,44 or a combination of these mechanisms. Ki-67, a marker of the G1, S, G2, and M phases of the cell cycle,45,46 was used to quantify smooth muscle cell proliferation. In accordance with previous studies using other proliferation markers, the Ki-67 immunohistochemistry demonstrated that the proliferation rate of the medial smooth muscle cells was still significantly elevated 2 weeks after angioplasty and that the mitotic activity in the intima was even more pronounced, particularly in the subendothelial region.47 The effects of L-arginine were surprising: it did not alter absolute or relative numbers of Ki-67-positive cells in the media, but elevated their number in the intima even more,48 particularly in the subendothelial region.47 The effects of L-arginine were surprising: it did not alter absolute or relative numbers of Ki-67-positive cells in the media, but elevated their number in the intima even more,48 particularly in the subendothelial region.47 The effects of L-arginine were surprising: it did not alter absolute or relative numbers of Ki-67-positive cells in the media, but elevated their number in the intima even more,48 particularly in the subendothelial region.47
not influenced by L-arginine demonstrates that smooth muscle cells in the media and intima display differences in sensitivity to L-arginine.

Whether perivascularly delivered L-arginine really reaches neointimal cells also remains speculative. Perhaps neointimal thickness is determined by other non-L-arginine related adventitial-neointimal interactions. Perivascular administration of L-NAME, at least in the dose we have chosen, did not have any detectable effect on neointima formation, on the number or the mitotic index of the smooth muscle cells in intima and media, or on the vascular remodeling. The dose (10 mg L-NAME \( \cdot \) kg\(^{-1} \cdot \) day\(^{-1} \)) and route of administration were selected based on the proven effectiveness of this drug to accelerate intimal thickening in hypercholesterolemic rabbits. However, the eNOS pathway remains active in that model, whereas functional studies indicate that iNOS activity prevails in rabbit carotid arteries after balloon dilatation. Although L-NAME (30 \( \mu \)mol/L) evoked endothelium-independent contractions in rabbit carotid arteries isolated after angioplasty, indicating that it inhibited vascular iNOS activity, the drug is 300-fold less sensitive to L-arginine. Hence, the dose may have been inadequate to suppress iNOS activity in vivo despite its effectiveness in a model of intimal thickening in which at least a significant part of the NO is formed by eNOS. Furthermore, it is conceivable that part of the L-NAME was metabolized to L-arginine, which could have counteracted the effects of L-NAME. Indeed, local perivascular application of L-NAME also failed to accelerate balloon-induced neointima formation in the rat carotid artery. Hence, the lack of effect of L-NAME in both models of balloon-induced intimal thickening and remodeling does not necessarily dismiss the hypothesis that NO formed by iNOS modulates these processes. Alternative doses and routes of administration certainly have to be considered to test the efficacy of this drug and, hence, to support the effect of L-arginine in this model of endothelial injury in nonatherosclerotic rabbits.

In conclusion, neointimal formation and biphasic vascular remodeling occur after experimental balloon dilatation in the nonatherosclerotic rabbit carotid artery, and can be influenced by continuous local perivascular delivery of L-arginine. Although the mechanisms underlying these beneficial effects deserve further investigation, these findings may have some clinical implications in the prevention of restenosis after PTCA.

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References


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