Effects of α-lipoic acid on ischemia-reperfusion-induced renal dysfunction in rats

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IN ACUTE KIDNEY INJURY (AKI), there is a wide range of glomerular and tubular abnormalities that contribute to the overall renal dysfunction. An ischemia-reperfusion (I/R) insult in rats is an AKI model that is widely used. In this model, there are structural alterations in renal tubules, in association with impaired urinary concentration and sodium reabsorption. We have previously demonstrated a diminished adenylyl cyclase activity and aquaporin-2 (AQP2) expression, along with a decreased expression of the type 3 Na/H exchanger (NHE3) and Na-K-ATPase in rat kidneys after an ischemic injury (16, 20). In this context, a modulation of AQP and sodium transporters could be a relevant therapeutic approach in I/R-induced AKI.

On the other hand, it has been known that reactive oxygen species (ROS) are powerful mediators of renal endothelial and tubular cell injury (31), and hence antioxidants may be potential candidates for prevention or treatment of I/R-induced renal injury. α-Lipoic acid (α-LA) has been known as a potent antioxidant, of which antioxidant effects are attributed to direct radical scavenging and metal chelation. However, whether α-LA can prevent the dysregulation of renal AQP and sodium transporters associated with I/R-induced renal injury has not been determined.

In AKI, mechanisms underlying cell morbidity are multifactorial and incompletely understood. Among others, intrarenal vasoconstriction, related to a shift in the balance between endothelin (ET) and nitric oxide (NO), is receiving considerable attention as a major contributor to the pathogenesis of AKI (7). The present study was aimed to determine whether treatment of α-LA prevents I/R-induced renal dysfunction and the dysregulation of AQP5 and sodium transporters. We also examined the effects of α-LA on the NO/cGMP and ET systems, which are the possible mediators of the renal tubular injury in I/R-induced AKI.

MATERIALS AND METHODS

Experimental protocols. Male Sprague-Dawley rats were used. The experimental procedure conformed to the Institutional Guidelines for Experimental Animal Care and Use.

The rats were divided into three groups: sham-operated control (n = 5), I/R injury (n = 6), and I/R injury combined with treatment with α-LA (n = 6). To induce I/R injury, both renal pedicles of the rats were clamped for 40 min under ketamine anesthesia (50 mg/kg ip). Control rats underwent a sham operation without clamping of the renal pedicel. α-LA (80 mg/kg, ip, Bukwang) was administered four times (at 48 and 24 h before the pedicle clamping and at 6 and 24 h after reperfusion). The same amount of physiological saline was injected for the control and I/R injury groups. The rats were maintained individually in metabolic cages to allow urine collections for the measurement of Na+, K+, creatinine, and osmolality. They were killed for semiquantitative immunoblotting and immunohistochemical studies 2 days after the clamping. Under anesthesia with ketamine, blood samples were collected from the inferior vena cava and analyzed for Na+, K+, creatinine, and osmolality. The right kidney was rapidly removed, dissected into three zones [cortex and outer stripe of outer medulla (cortex/OSOM), inner stripe of outer medulla (ISOM) and inner medulla (IM)], and processed for immuno-
As described below. The left kidney was fixed by retrograde perfusion.

In another set of experiments, the rats were decapitated while in a conscious state and the right kidney was removed and processed to determine enzyme activities. The left kidney was also taken and kept at -70°C until assayed for the mRNA expression by real-time PCR.

In a third set of experiments, the effects of -LA alone on the expression of AQPs and sodium transporters were examined in control rats. The rats were treated with -LA as above without clamping of the renal pedicles. The kidneys were taken and processed for immunoblotting of AQP and sodium transporters.

Semiquantitative immunoblotting. The dissected cortex/OSOM, ISOM, and IM were homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1% Triton X-100, 1 mM EDTA, 8.5 M leupeptin, and 1 mM phenylmethylsulfonyl fluoride, with pH 7.4. The homogenates were centrifuged at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. SDS-PAGE was performed on 9 or 12% polyacrylamide gels. The proteins were transferred by gel electrophoresis (Mini Protean II, Bio-Rad) onto nitrocellulose membranes (Amersham Pharmacia Biotech, Hybond ECL RPN3032D; Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBST (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated blotting as described below. The left kidney was fixed by retrograde perfusion.

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AQP3 expression in I/R rats, which was reversed by 29- and 35- to 50-kDa AQP3 bands, representing nonglycosylated and glycosylated forms of AQP3, respectively. Densitometric analysis revealed a decreased

Fig. 2. A: semiquantitative immunoblotting of AQP3 in the membrane fraction of ISOM. The immunoblot was reacted with affinity-purified anti-AQP3, revealing 29- and 35- to 50-kDa AQP3 bands, representing nonglycosylated and glycosylated forms of AQP3, respectively. Densitometric analysis revealed a decreased AQP3 expression in I/R rats, which was reversed by α-LA treatment. *P < 0.05 compared with control. #P < 0.05 compared with I/R group.

B: immunoperoxidase microscopy of AQP3 in OMCD. AQP3 labeling was present at the basolateral membrane domains of the collecting duct in control, I/R and I/R+α-LA-treated rats. There was a decreased AQP3 immunolabeling in OMCD in I/R rats, which was reversed by α-LA treatment. Magnification: ×400.
Protein concentrations were measured by a bicinchoninic acid assay kit (Bio-Rad).

Adenylyl cyclase activity. Adenylyl cyclase activity was assayed by the method of Bar (2) with a slight modification. Protein samples were provoked by AVP. The reaction was started by adding the membrane fraction to 100 µl of working solution (50 mM Tris·HCl, pH 7.6, containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl2, 1 mM 3-isobutyl-1-methylxanthine, and 0.02 mM GTP). After 15 min, the reaction was stopped by cold application of a solution consisting of 50 mM sodium acetate, pH 5.0, and centrifuged at 1,000 g for 10 min at 4°C. cAMP was measured in the supernatant by equilibrated radioimmunoassay as previously described (20). Results are expressed as picomoles cAMP per milligram protein per minute.

sGC activity. SGC activity was measured using the soluble fraction of the papilla. The protein samples were incubated for 15 min at 37°C in 50 mM Tris·HCl (pH 7.6) containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl2, 1 mM 3-isobutyl-1-methylxanthine, and 0.02 mM GTP. After 15 min, the reaction was stopped by adding ice-cold 50 mM sodium acetate (pH 5.0) and boiling for 5 min. Samples were then centrifuged (10,000 g for 10 min at 4°C), of which the supernatant was used to measure cGMP by equilibrated radioimmunoassay (21). Results are expressed as picomoles cGMP per milligram protein per minute.

Colorimetric assay of nitrite/nitrate. As an index of synthesis of NO, its stable metabolites (nitrite/nitrate; NOx) were measured in urine by a colorimetric NO assay kit (Oxford Biochemical, Oxford, MI). A microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrate with Griess reagent, 80 µl MOPS (50 mM)/EDTA (1 mM) buffer and 5-µl urine samples were added to wells. Nitrate reductase (0.01 U) and 10 µl NADH (2 mM) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Color reagents, sulfanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride were added, and absorbance values were read at 540 nm in a microtiter plate reader (model 3550, Bio-Rad). NOx concentrations were estimated from a standard curve that was constructed with the use of standard reagents included in the assay kit.

Statistical analyses. Results are expressed as means ± SE. Multiple comparisons among the groups were made by one-way ANOVA and a post hoc Tukey honestly significant difference test. P values <0.05 were considered significant.

RESULTS

Renal function. I/R injury decreased creatinine clearance and increased plasma creatinine levels (Table 1). Accordingly, urine output significantly increased, in association with a marked reduction of its osmolality. In addition, the fractional excretion of sodium increased significantly, suggesting an impaired tubular sodium reabsorption in postischemic kidneys. The treatment with α-LA lowered plasma creatinine levels and increased creatinine clearance compared with those in untreated I/R rats. It reduced the degree of polyuria, in association with increased urine osmolality compared with that of untreated I/R rats. It also normalized the fractional excretion of sodium.

Expression of AQP5s. Semiquantitative immunoblotting revealed decreased abundance of AQP2 proteins in the cortex/inner medulla in control, I/R and I/R + α-LA-treated rats. The immunoblot was reacted with anti-type VI adenylyl cyclase. Densitometric analysis revealed a decreased expression in I/R rats, which was prevented by α-LA treatment. *P < 0.05 compared with control. #P < 0.05 compared with I/R group. □, Control; ●, I/R; ■, α-LA treated rats. Each point represents the mean ± SE of experimental rats.

Table 3. Protein expression of major sodium transporters

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>I/R (n = 4)</th>
<th>I/R + α-LA (n = 5)</th>
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<tbody>
<tr>
<td>Cortex/OSOM</td>
<td></td>
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<tr>
<td>Na-K-ATPase</td>
<td>1.00 ± 0.08</td>
<td>0.32 ± 0.12*</td>
<td>0.63 ± 0.13**†</td>
</tr>
<tr>
<td>NHE3</td>
<td>1.00 ± 0.15</td>
<td>0.28 ± 0.06*</td>
<td>1.32 ± 0.34†</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.00 ± 0.17</td>
<td>0.38 ± 0.07*</td>
<td>0.87 ± 0.06†</td>
</tr>
<tr>
<td>NCC</td>
<td>1.00 ± 0.10</td>
<td>0.50 ± 0.07*</td>
<td>0.65 ± 0.09*</td>
</tr>
<tr>
<td>ISOM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na-K-ATPase</td>
<td>1.00 ± 0.07</td>
<td>0.13 ± 0.01*</td>
<td>0.43 ± 0.19†</td>
</tr>
<tr>
<td>NHE3</td>
<td>1.00 ± 0.13</td>
<td>0.83 ± 0.33*</td>
<td>1.38 ± 0.34†</td>
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<tr>
<td>NKCC2</td>
<td>1.00 ± 0.24</td>
<td>0.22 ± 0.04*</td>
<td>0.80 ± 0.24†</td>
</tr>
<tr>
<td>Inner medulla</td>
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</tr>
<tr>
<td>Na-K-ATPase</td>
<td>1.00 ± 0.17</td>
<td>0.39 ± 0.06*</td>
<td>0.53 ± 0.10*</td>
</tr>
</tbody>
</table>

Values are means ± SE. NHE3, type-3 Na-H exchanger; NKCC2, bumetanide-sensitive Na-K-2Cl cotransporter; NCC, thiazide-sensitive sodium chloride cotransporter. *P < 0.05 compared with control. †P < 0.05 compared with I/R group.
cortex/OSOM and IM (Fig. 1, Table 2). Immunoperoxidase microscopy revealed decreased immunolabeling of AQP2 in the cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD) in I/R-injury rats, which was most pronounced in the OMCD. Being consistent with the immunoblotting data, the collecting duct AQP2 immunolabeling was markedly attenuated in response to α-LA treatment, in which the residual AQP2 was

![Image](image1)

**Fig. 4.** A: semiquantitative immunoblotting of α1-subunit of Na-K-ATPase in the membrane fraction of ISOM. Immunoblot was reacted with an anti-α1-subunit of Na-K-ATPase. Densitometric analysis revealed that the protein expression of the α1-subunit of Na-K-ATPase was decreased in I/R rats, which was counteracted by α-LA treatment. *P < 0.05 compared with control. #P < 0.05 compared with I/R group. B: immunoperoxidase microscopy of Na-K-ATPase in ISOM. Immunolabeling was associated with basolateral plasma membrane of the thick ascending limb (TAL). Decreased immunolabeling was shown in I/R rats, which was reversed by α-LA treatment. Magnification: ×200.

![Image](image2)

**Fig. 5.** A: semiquantitative immunoblotting of Na-K-2Cl cotransporter (NKCC2) in the membrane fraction of ISOM. The immunoblot was reacted with anti-NKCC2. Densitometric analysis revealed decreased expression of NKCC2 in I/R rats. α-LA treatment partially prevented the downregulation of NKCC2 in I/R rats. *P < 0.05 compared with control. #P < 0.05 compared with I/R group. B: immunoperoxidase microscopy of NKCC2 labeling was associated with apical plasma membrane of the TAL in control rats. Immunoperoxidase microscopy demonstrated decreased NKCC2 immunolabeling in TAL in I/R rats. In contrast, α-LA treatment partially recovered NKCC2 immunolabeling in I/R rats. Magnification: ×200.
located not only in the cytoplasmic region but also in the apical membrane of the principal cell (Fig. 1).

The expression of AQP3 was also decreased in I/R rats, which was partially reversed by the treatment with α-LA in the ISOM. Immunoperoxidase microscopy demonstrated decreased AQP3 immunolabeling in the collecting duct in I/R rats, which was partially reversed by α-LA treatment (Fig. 2).

The expression of AQP1 was significantly decreased in I/R rats, which was reversed by the treatment of α-LA (Table 2). Immunohistochemistry confirmed a decreased AQP1 labeling in proximal tubules of rats with I/R, which was prevented by the treatment with α-LA (data not shown).

Enzyme activity and protein expression of adenylyl cyclase. Figure 3A shows the immunoblots of type VI adenylyl cyclase, the major isoform expressed in the collecting duct (6). Its antibody recognized a broad band around 160 kDa, of which expression was decreased in the ISOM in I/R-injury rats, which was counteracted by the treatment with α-LA in the ISOM. Figure 3B shows cAMP generation in response to graded doses of AVP. The AVP-stimulated cAMP generation was blunted in I/R rats, which was then ameliorated by α-LA treatment.

Expression of sodium transporters. The protein expression of Na-K-ATPase α1-subunit was markedly decreased in the cortex/OSOM, ISOM, and inner medulla in I/R rats. α-LA treatment prevented the downregulation of Na-K-ATPase in the cortex/OSOM and ISOM (Table 3, Fig. 4A). Immunohistochemistry also revealed a decreased AQP1 labeling in proximal tubules of rats with I/R, which was prevented by the treatment with α-LA (data not shown).

The protein expression of NKCC2 was markedly decreased in the cortex/OSOM and ISOM in I/R rats, which was prevented by α-LA treatment (Table 3, Fig. 5A). Immunohistochemistry also revealed an overall decrease in NKCC2 labeling in the apical domains of the TAL in control rats. α-LA-treatment restored the NKCC2 labeling in the TAL (Fig. 5B). Moreover, the expression of NHE3 was decreased in I/R rats, which was restored to the control level by α-LA treatment in the cortex/OSOM and ISOM. In contrast, the expression of NCC was decreased in I/R rats, however, not being affected further by α-LA treatment (Table 3).

Expression of ET-1. Figure 6 shows mRNA expression of ET-1, ETAR, and ETBR. The expression of ET-1 was increased by I/R injury, which was attenuated by α-LA treatment. In contrast, the expression of ETAR decreased in I/R rats, which was recovered to the control level by α-LA treatment. ETBR showed no significant differences among the three groups.

NOS expression and urinary NO metabolites. Figure 7 shows the protein expression of eNOS, nNOS, and iNOS in ISOM. The expression of eNOS and nNOS was decreased, while that of iNOS was increased in I/R rats, which was counteracted by the treatment with α-LA (Table 4). The amount of urinary NOx excretion was decreased in I/R injury (2.86 ± 0.28 in I/R vs. 7.69 ± 0.15 mmol in control, P < 0.05), which was ameliorated by α-LA treatment (18.9 ± 0.34 mmol, P < 0.05).
Enzyme activity and expression of sGC. sGC α₁-subunit antibody recognized a broad band around 72 kDa, of which expression was decreased in IM in I/R-injury rats, which was counteracted by the treatment with α-LA (Fig. 8A). The cGMP generation in response to graded doses of SNP was blunted in I/R rats, which was prevented by α-LA treatment (Fig. 8B).

Expression of renal AQPs and sodium transporters in rats treated with α-LA alone. α-LA treatment alone did not affect the expression of AQP1 (data not shown), AQP2, AQP3 (data not shown), the α₁-subunit of Na-K-ATPase or NHE3 in the ISOM (Fig. 9).

DISCUSSION

We have previously demonstrated the downregulation of AQP and major sodium transporters, along with impaired urinary concentrating ability and renal sodium handling in I/R-induced AKI (16, 20). The present study further demonstrated that α-LA treatment prevented the dysregulation of AQP and sodium transporters and reserved the urinary concentrating ability and tubular sodium reabsorption capability despite the I/R injury.

It has been well known that AVP plays a regulatory role in the expression of AQP2 and AQP3 (38). In the present study, cAMP generation in response to AVP was blunted in I/R rats.

Fig. 8. A: semiquantitative immunoblotting of α₁-subunit of soluble guanylyl cyclase (sGC) in the cytosolic fraction of IM. The immunoblot was reacted with the anti-α₁-subunit of sGC. Densitometric analysis revealed a decreased expression in I/R rats, which was prevented by α-LA treatment. *P < 0.05 compared with control. #P < 0.05 compared with I/R group. B: cGMP production in response to sodium nitroprusside (SNP) in inner medullar. ○, Control; ●, I/R; ■, α-LA treated rats. Each point represents the mean ± SE of experimental rats.

Fig. 9. Semiquantitative immunoblotting of AQP2, α₁-subunit of Na-K-ATPase, and type 3 Na/H exchanger (NHE3) from membrane fraction of ISOM. The immunoblot was reacted with anti-AQP2, the anti-AQP2 and anti-NHE3, respectively. Densitometric analyses show no significant changes among the groups.

Accordingly, the expression of type VI adenylyl cyclase was significantly decreased, to which the blunted production of cAMP may be attributed. Similarly, immunohistochemistry revealed decreases in AQP2 immunoreactivities in the principal cell of the collecting duct in I/R-induced AKI. However, the subcellular localization of AQP2 was preserved despite the reduced abundance. α-LA treatment reserved the expression of adenylyl cyclase and production of cAMP. The improved cAMP generation by α-LA treatment may have contributed to the normalized regulation of AQP2 and AQP3 channels.

The abundance of several sodium transporters (Na-K-ATPase, NHE3, NKCC2, and NCC) was severely reduced in response to I/R injury, which was most pronounced in the ISOM. Immunoperoxidase microscopy also showed a dramatic decrease in Na-K-ATPase labeling in the proximal tubule and TAL. Furthermore, α-LA treatment prevented the I/R-induced downregulation of sodium transporters and maintained fractional sodium excretion. The decreased abundance of sodium transporters in postischemic kidneys may have impaired tubular sodium reabsorption in AKI. The decrease in NHE3 and NKCC2 may have resulted in activation of the
tubulogglomerular feedback mechanism, and hence decreased GFR. The reduced GFR in turn may play a role in preventing the extensive loss of urinary sodium that could otherwise occur in postischemic kidneys.

I/R-induced AKI is associated with prolonged vasoconstriction, so that functional recovery may be prevented or delayed (17). Recent observations have indicated that ET and NO systems have a crucial role in the pathogenesis of ischemic AKI (29, 35). In the present study, the expression of ET-1 mRNA was increased following I/R injury, which was attenuated by α-LA treatment. These findings are in line with recent observations (37), in which ET-1 content was markedly increased in the kidney after I/R, which was attenuated by α-LA treatment. Furthermore, it has been found that oxidative stress stimulates renal ET-1 production (19) and antioxidants could suppress the ET-1 production in renal and vascular endothelial cells (3, 19, 33). It is likely that α-LA can suppress the I/R-induced ET-1 overproduction through its antioxidative activity. In addition, the mRNA expression of ETαR decreased in I/R rats, which was recovered to the control level by α-LA treatment. Exposure of cultured smooth muscle cells (18) and glomerular mesangial cells to ET-1 markedly decreases 125I-ET-1 binding sites, indicating an autologous downregulation of ET receptors (40). The downregulation of ETαR may play a compensatory role in renal vasoconstriction induced by ET-1 in I/R-induced AKI. In the collecting duct, ET-1 inhibits AVP-induced increases in water permeability and cAMP accumulation via ETβR (12). Interestingly, however, ETβR expression was not affected despite the enhanced ET-1 synthesis in the present study. It is conceivable that increased activity of ET-1/ETβR may decrease the adenyl cyclase activity and AQP2 expression in the collecting duct in I/R-induced AKI, which could be partly reversed by α-LA treatment.

NO plays an important role in regulating various renal functions (35). However, the role of NO in I/R-induced AKI has not been established. N⁵-nitro-L-arginine methyl ester prevented hypoxia-regeneration injury in isolated rat proximal tubules (44). It was also found that SNP or L-arginine, a NO precursor, enhanced the injury (44). In contrast, the inhibition of NO production with N⁵-nitro-L-arginine significantly deteriorated renal function of postischemic kidney in rats (8). The conflicting data have been attributed to different isoforms of NO. eNOS and nNOS are localized in the vascular endothelium and macula densa (1), whereas iNOS is present in the proximal tubule and IMCD (28). iNOS may be a key mediator of inflammatory processes such as in septic shock (41) and I/R injury (10). It has been recently shown that eNOS leads to restoration of renal function after an injury, while activation of iNOS leads to excessive NO production and aggravation of renal failure (32). In the present study, iNOS expression was increased in I/R-induced injury, while eNOS and nNOS expression decreased. These findings suggest that an imbalance between the activities of iNOS and constitutive NO (eNOS and nNOS) is an important contributor to the I/R-induced renal injury. Increased NO production by iNOS may be responsible for a cytotoxic injury resulting in I/R-induced AKI. Along with the decreased expression of eNOS and nNOS, the urinary NOx excretion was decreased in I/R injury, which was counteracted by the α-LA treatment. Considering that the increase in iNOS was modest (20%), while the decrease in eNOS (59%) and nNOS (88%) expression was rather prominent in I/R-induced AKI, the decreased renal production of NO may be attributed to decreased eNOS and nNOS expression. The decreased expression of constitutive NO may be causally related to decreases in renal blood flow, which may aggravate the ischemic renal damage combined with increased synthesis of ET. The biological action of NO is mediated by activation of cytosolic guanylyl cyclase and secondary generation of cGMP (14). The SNP provoked cGMP generation was decreased in I/R rats, in association with decreased expression of sGC proteins, which was in turn prevented by α-LA treatment. This finding suggests that α-LA is capable of reversing changes in the NO/cGMP system and may therefore be an important therapeutic option to prevent renal damage in I/R-induced AKI.

It has been shown that α-LA has direct cellular effects of prooxidant (4) and AMP kinase activation (25). This would indicate a possible direct effect of α-LA. However, in the present study, α-LA alone did not affect the abundance of AQP5 and sodium transporters. These results suggest that α-LA treatment counteracts the effects of I/R injury, when applicable, possibly through reversing the dysregulation of ET and NO systems rather than its direct effect.

**Conclusion.** The ischemic insult resulted in downregulation of AQP and major sodium transporters in postischemic kidneys, coinciding with the impairment of urinary concentration and decreased tubular reabsorption of filtered sodium. α-LA treatment has a protective effect against renal I/R injury, such as normalizing the renal hemodynamics, urinary concentration ability, and excretion of sodium, along with the expression of AQP and sodium transporters. α-LA treatment also restored the regulation of the ET system and the abundance of various NO isoforms. The protective effect of α-LA in I/R-induced AKI may be attributed to preservation of normal activities of local AVP/cAMP, NO/cGMP, and ET systems.

**GRANTS**

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