REGULAR PAPER

J. Physiol. Sci. Vol. 57, No. 3; Jun. 2007; pp. 187–192
Online Jun. 5, 2007; **doi:10.2170/physiolsci.RP004707**

Effect of Chronic Stress and L-Carnitine on Rat Stomach

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Abstract: Background and Aim: L-Carnitine is an essential cofactor in the mitochondrial transfer of fatty acids, and it is also a scavenger of free radicals in mammalian tissues. The aim of the study was to determine the effect of L-carnitine on chronic restraint stress–induced gastric mucosal injury. Methods: Wistar rats were applied restraint stress (1 h/day) and L-carnitine (50 mg/kg) for 21 days. The lesion index, prostaglandin E_2 and mucus content, lipid peroxidation, superoxide dismutase, and catalase activity in gastric mucosa were evaluated. Results: Chronic restraint stress increased the lesion index, lipid peroxidation, and superoxide dismutase activity in gastric mucosa, and it decreased prostaglandin E_2 and mucus content. L-Carnitine treatment prevented the stress-induced increase in lesion index, lipid peroxidation and a stress-induced decline in prostaglandin E_2, and mucus content in gastric mucosa, but it increased catalase activity. Conclusions: L-Carnitine prevents the occurrence of lesion by strengthening the gastric mucosal barrier and by reducing lipid peroxidation against the harmful effects of chronic restraint stress.

Key words: restraint stress, L-carnitine, lipid peroxidation, gastric injury.

Stress has been postulated to be involved in the etiopathogenesis of a variety of disease states, including hypertension, diabetes, depression of immune system, reproductive dysfunctions, peptic ulcers, and behavioral changes like anxiety because of an involvement of the central nervous system and the endocrine system [1–3]. The disturbances may vary by type, intensity, and duration of a stressor, and the strain/sex differentiation of the experimental subjects [3]. Restraint stress is widely used as an acute and chronic stress model [4–6]. The length of stress period may alter important neurological, behavioral, and biochemical parameters, possibly in different ways [7].

It has been shown that exposure to stress can stimulate numerous pathways leading to an increased production of free radicals [3, 7, 8]. It is well known that free radicals activate a cascade, producing lipid peroxidation, protein oxidation, DNA damage, and cell death, and that they contribute to the occurrence of pathological conditions [9, 10]. Stress may also impair the antioxidant defense system, leading to oxidative damage, by changing the balance between oxidant and antioxidant factors [8, 11, 12]. Previous studies have reported that various situations of stress induces lipid peroxidation measured in tissues followed by decreased activities of the antioxidant enzymes and increased production of reactive oxygen species [11–13].

Clinical experience suggests that various types of stress, such as shock, burns, sepsis, and severe trauma, play roles in the induction of acute gastric mucosal lesions. The restraint of rats is commonly used to study the pathogenesis of stress-induced gastric injury [8–11, 14]. Restraint stress has been shown to induce the production of oxygen-derived free radicals in the stomach, which leads to mucosal ulceration, even in the absence of luminal acid [8–11]. Preventing the formation of free radicals or enhancing their disposal with scavengers such as superoxide dismutase alleviates the mucosal damage provoked by stress [7, 8, 10, 14].

L-Carnitine is a natural substance that acts as a carrier of fatty acids across the inner mitochondrial membrane for subsequent beta-oxidation. It has an antiperoxidative effect on several tissues [15–19], which may account for the beneficial effect in stress-induced injury. It protects cells from oxidative stress by at least two mechanisms, preventing the formation of oxygen reactive species through the xanthine oxidase–xanthine system and decreasing the damage to membrane lipids and proteins. It also has a scavenger effect on reactive oxygen species and a stabilizing effect on damaged cell membranes [20].

The antioxidant properties of L-carnitine have been established. Moreover, L-carnitine's effects on stomach in rats exposed to chronic restraint stress have not as yet been evaluated. In the present study we have investigated the protective effect of L-carnitine on stomach in a chronic stress model.

Received on May 10, 2007; accepted on Jun 1, 2007; released online on Jun 5, 2007; doi:10.2170/physiolsci.RP004707
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MATERIALS AND METHODS

Sixty-two male Wistar rats, weighing 200–250 g were used. The animals were housed at 22 ± 1°C on a 12-h day-night regimen and received a standard diet and water ad libitum. The study protocol was approved by the Akdeniz University Laboratory Animals Maintenance and Usage Committee.

Preparation of animals. The rats were randomly divided into four groups. Group 1 (control rats; $n = 10$) received 1 ml of distilled water orally by intragastric gavage every day for 21 days. Group 2 (stressed rats; $n = 15$) received 1 ml of distilled water by intragastric gavage and was restrained for 1 h daily for 21 days at 4°C by placing individual animals in 25 × 7 cm plastic cages. Group 3 (carnitine-treated rats; $n = 10$) received L-carnitine (50 mg/kg) suspended in 1 ml of distilled water by intragastric gavage daily for 21 days. Group 4 (carnitine-treated and stressed rats; $n = 15$) received L-carnitine (50 mg/kg) suspended in 1 ml of distilled water by intragastric gavage and restrained for 1 h daily for 21 days at 4°C by placing individual animals in 25 × 7 cm plastic cages.

The animals were deprived of food for 24 h prior to the experiments, but were allowed free access to water. The animals were anesthetized intraperitoneally with 1 g urethane/kg and killed by exsanguination via the abdominal aorta.

Determination of gastric lesions. The stomachs were removed rapidly, opened by an incision along the greater curvature, and pinned onto a platform. The lesions were counted and measured with a stereomicroscope (Zeiss Stemi SV 11, magnification ×6, Carl Zeiss Microscope, Zeiss Group, D-07740 Jena, Germany) by an observer who was blinded to the treatment. The sum of the lesion areas in each animal as mm^2 was expressed as the mean gastric mucosal injury.

Measurement of gastric acidic mucopolysaccharides. Acidic mucopolysaccharides, as an indicator of the gastric mucosal barrier, were measured based on the Alcian blue binding capacity of gastric mucosa. One half of every stomach was used for acidic mucopolysaccharide analysis according to the method of Corne *et al.* [21].

Stomach tissue was everted immediately and rinsed in ice-cold 0.25 M sucrose solution. It was weighed and soaked for 2 h in 10 ml 0.1% Alcian blue 8 Gx, dissolved in 0.16 M sucrose, buffered with 0.05 M sodium acetate, and adjusted to pH 5.8 with HCl. Each tissue was transferred to 10 ml of 0.25 M sucrose, and two successive washes of 15 and 45 min in 0.25 M sucrose were carried out. The dye complexed with mucus was then eluted by immersion for 2 h in aliquots of 0.5 M $MgCl_2$ 10 ml/g of tissue occasionally shaken, removed, and the magnesium chloride solution was shaken briefly with 10 ml diethyl ether. The optical density of the aqueous layer was read at 605 nm. Results were expressed as μg per gram of tissue.

Determination of gastric PGE_2 level. The assay of gastric PGE_2 levels was performed according to the method described by Cockrell and Ellis [22]. PGE_2 content of the gastric mucosa was measured using a Varian HPLC (model 5000; Varian Instrument Group, Walnut Creek, CA). Gastric mucosa was scraped rapidly with a glass slide. The scrapings were weighed and placed in a Kreb's solution containing 128 mM NaCl, 4.7 mM KCl, 3.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 14.3 mM $NaHCO_3$, 11.1 mM glucose, and 28 μM indomethacin (to prevent any neoformation of PG during the extraction process). The tissue sample was then homogenized with tissue homogenizator (TRI-R, STIR-R, model K43) for 20 s under ice-cold conditions, followed by centrifugation at 15,000 × *g* for 20 min at 4°C. The supernatant was stored at –20°C until the assay (Ko and Cho, 1999). A sample of 2 ml from each supernatant was added to 2 ml chloroform, and rinsed for 1 min. The extracts were evaporated under the nitrogen and dissolved in 1 ml of mobile phase composed of water:acetonitril:benzene:acetic acid (767:230:2:1, v/v/v/v), and loaded onto a reverse phase column, SP C18 (150 × 4 mm, 3 μm particle size). Mucosal extracts were eluted with a mobile phase flow rate of 1 ml/min. The peaks were detected by UV absorbance at 280 nm at 28°C. A quantitative integration of chromatographic separations was performed by using a Varian integrator (model 4290; Varian Instrument Group, Walnut Creek, CA) and PGE_2 standard as a reference.

Estimation of lipid peroxidation in gastric mucosa. To measure thiobarbituric acid reactant substances (TBARS) content in the gastric mucosa, an index of lipid peroxidation, the mucosa of the stomach was scraped and frozen on dry ice with a tissue disrupter (TRI-R STIR-R, Model K43, TRI-R Instruments, Rockville Centre, NY, USA) driven at 4,000 rpm for 30 s two times. Mucosal homogenates at a final concentration of 10% (w/v) were prepared in homogenization fluid, and TBARS content was measured in homogenates by the thiobarbituric acid method of Stocks and Dormandy [23, 24]. 1,1,3,3-Tetraethoxypropane (Sigma Chemical, St. Louis, MO, USA) was used as the standard for calibration of the curve. The results were expressed as picomoles of malondialdehyde per gram protein. Proteins were determined by Lowry's method.

Assay of gastric mucosal antioxidant enzymes. Superoxide dismutase (SOD) activity in the gastric mucosa was assayed by the method described by Misra *et al.* [25]. Mucosal tissue was homogenized at 9,000 rpm for 30 s. Homogenates were centrifuged at 25,000 rpm for 60 min at 4°C. Supernatants were added to the reaction mixture (550 μl HCO_3 buffer, 400 μl EDTA, 500 μl Epinephrine), and SOD activity was measured at 480 nm. Adrenochrome was generated with epinephrine autooxidation. Since the production of adrenochrome is inhibited by Cu/Zn SOD, Cu/Zn SOD is responsible from the change of absorbance at 480 nm, at which the absorbance of adreno-

chrome is maximum. SOD activity was expressed as the amount of the SOD standard showing activity equivalent to the determined activity. The results have been expressed as units (U) of SOD activity/g protein.

Catalase activity of gastric mucosa was assayed by the method of Aebi *et al.* [26]. The collected mucosal tissue was disrupted using the homogenizer in 50 mM phosphate buffer (pH 7.00), and the prepared homogenate was centrifuged at 5,000 rpm for 15 min at 4°C. The principle of the assay is based on the determination of the rate constant (k, s^{-1}) or the decomposition rate of H_2O_2 at 240 nm. The results were expressed as k per gram protein (k, g protein^{-1}).

Statistics. The presence of significant differences between mean values was determined by the analysis of variance (ANOVA) followed by Tukey's HSD test post hoc test. Each value is the mean ± SE from at least 10 experiments.

RESULTS

Gastric mucosal injury

To further confirm the dose-dependent effect of L-carnitine on chronic stress–induced gastric mucosal injury formation in rats, the different doses of L-carnitine (10, 50, or 100 mg/kg) were given orally for 21 days associated with stress. We found that there was a protective effect at concentrations of more than 50 mg/kg per day. However, no significantly different effect was observed between 50 and 100 mg/kg per day of L-carnitine (Table 1).

As seen in Fig. 1, neither distilled water alone nor L-carnitine alone produced any macroscopic lesions in the rat stomachs. Chronic restraint stress results in an increase in gastric epithelial cell loss and macroscopic mucosal injury ($p < 0.001$). However, pretreatment with L-carnitine inhibited the stress-induced gastric damage ($p < 0.05$ vs. the value in stressed rats).

Acidic mucopolysaccharide of gastric wall

The Alcian blue binding capacity of the gastric mucosa, which is accepted as the criterion of acidic mucopolysaccharide in gastric mucin, was 49.35 ± 3.75 μg/g wet wt in control rats. In rats exposed to chronic restraint stress, acidic mucopolysaccharide was significantly reduced to 18.52 ± 1.46 μg/g ($p < 0.001$). However, there was a significant increase in gastric mucus observed following L-carnitine administration in stressed rats ($p < 0.01$ vs. the value in stressed rats) (Fig. 2).

Table 1. The dose-dependent effects of L-carnitine on gastric mucosal lesion formation in rats exposed to 1 h of restraint stress at 4°C for 21 days.

	Lesion index (mm^2)
Control	0
Stress	3.42 ± 0.69
Stress + L-carnitine (10 mg/kg)	2.98 ± 0.74
Stress + L-carnitine (50 mg/kg)	1.83 ± 0.46*,#
Stress + L-carnitine (100 mg/kg)	1.52 ± 0.65*,#

Results are presented as mean ± SE of groups. *$p < 0.05$, vs. the value in stressed rats. #$p < 0.05$, vs. the value in the experimental group that received 10 mg/kg per day L-carnitine.

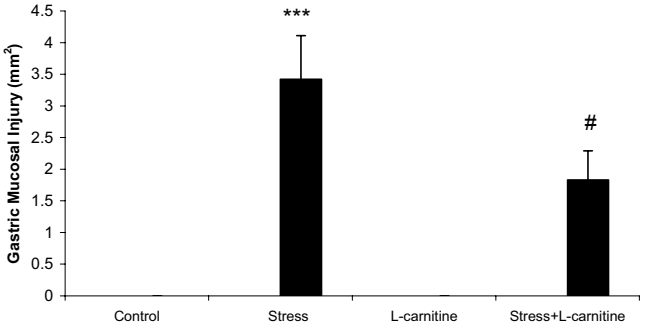


Fig. 1. Effect of L-carnitine on gastric mucosal injury induced by restraint stress for 21 days. L-Carnitine (50 mg/kg) was administered for the same period. The mucosal injury was expressed as the total area of mucosal erosions. Each bar represents means ± SE. ***$p < 0.001$ vs. the value in control rats; #$p < 0.05$ vs. the value in stressed rats.

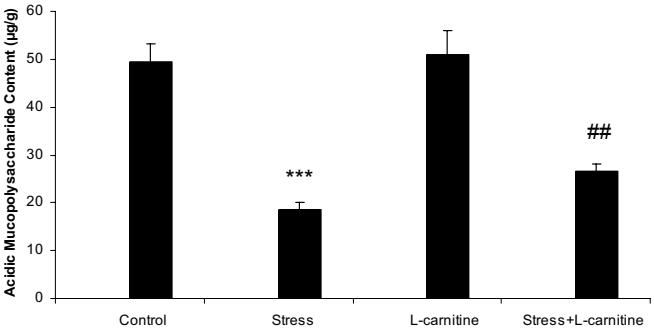


Fig. 2. Effect of L-carnitine on acidic mucopolysaccharide content of gastric mucosa in rats subjected to restraint stress for 21 days. L-Carnitine (50 mg/kg) was administered for the same period. Acidic mucopolysaccharide content was determined as described in MATERIALS AND METHODS. Each bar represents means ± SE. ***$p < 0.001$ vs. the value in control rats; ##$p < 0.01$ vs. the value in stressed rats.

PGE_2 content of gastric mucosa

The PGE_2 content of gastric mucosa in control rats (118.42 ± 23.32 pg/g) was not influenced significantly with L-carnitine treatment (110.13 ± 15.30 pg/g). Chronic restraint stress significantly reduced the PGE_2 content of gastric mucosa (53.38 ± 11.38 pg/g, $p < 0.01$ vs. the value in control rats). Treatment with L-carnitine prevented the decrease of mucosal PGE_2 (94.35 ± 19.71, $p < 0.05$ vs. the value in stressed rats) (Fig. 3).

Lipid peroxidation in gastric tissue

As seen in Table 2, TBA reactive substances in the gastric mucosa, the index of lipid peroxidation increased markedly from the basal concentration of 81.95 ± 7.50

Table 2. The effects of L-carnitine on stress-induced lipid peroxidation products and antioxidant enzyme activities.

	Control	Stress	L-Carnitine	Stress and L-carnitine
TBARS production (pmol/g protein)	81.95 ± 7.50	109.09 ± 9.80*	78.32 ± 8.61	79.86 ± 6.83$^{\#}$
SOD activity (U/g protein)	2.80 ± 0.26	7.09 ± 0.66***	2.98 ± 0.34	6.58 ± 0.29
Catalase activity (k/g protein)	27.58 ± 2.39	29.80 ± 2.65	59.84 ± 6.97^{+++}	50.94 ± 4.87$^{\#\#}$

Results are presented as mean ± SE of groups. *$p < 0.05$, ***$p < 0.001$, $^{+++}p < 0.001$ vs. the value in control rats. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the value in stressed rats.

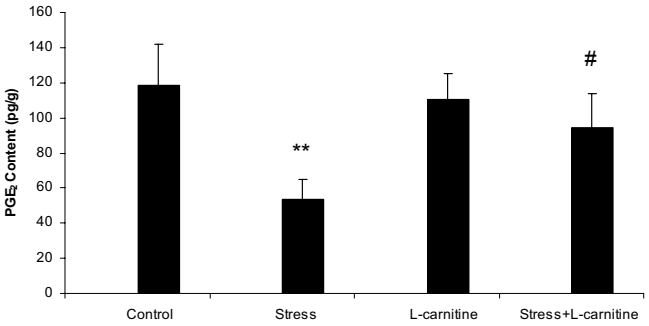


Fig. 3. Effect of L-carnitine on PGE_2 content of gastric mucosa in rats subjected to restraint stress for 21 days. L-Carnitine (50 mg/kg) was administered for the same period. PGE_2 content was determined as described in MATERIALS AND METHODS. Each bar represents means ± SE. **$p < 0.01$ vs. the value in control rats; $^{\#}p < 0.05$ vs. the value in stressed rats.

pmol/g protein to 109.79 ± 9.8 pmol/g protein in the gastric mucosa of stressed rats ($p < 0.05$). L-Carnitine administration significantly prevented the increase in lipid peroxidation products in the stressed groups ($p < 0.05$).

Activity of gastric antioxidant enzymes

Chronic restraint stress significantly increased SOD activity ($p < 0.001$), and did not change the catalase activity in the gastric tissue compared to the control values. L-Carnitine administration markedly increased catalase activity ($p < 0.01$), and was not effective in SOD activity in rats exposed to chronic restraint stress (Table 2).

DISCUSSION

The objective of this study was to assess the effect of chronic stress on the induction of oxidative gastric mucosal injury and the protective ability of L-carnitine. Wistar rats were subjected to repeated restraint stress for 21 days, after which the generation of lipid peroxidation, the situations of antioxidant enzymes, and mucosal damage in the gastric mucosa were determined. Furthermore, the gastroprotective ability of L-carnitine was assessed.

Carnitine is a vitaminlike substance and commonly obtained from diet. It is demonstrated that it can also be synthetized endogenously by skeletal muscle, heart, liver, kidney, and brain from the essential amino acids lysine and methionine [27]. There are many reports about its antioxidant effects against oxidative stress in tissues [15–19].

It has been shown that exposure to stress conditions can stimulate numerous pathways leading to an increased production of free radicals [8, 10, 11]. It is well known that free radicals generate a process, producing lipid peroxidation, protein oxidation, DNA damage, and cell death, and they contribute to the occurrence of pathological conditions [28, 29]. Stress may also impair the antioxidant defense system, leading to oxidative damage, by changing the balance between oxidant and antioxidant factors. Immobilization stress is followed by an increase in lipid peroxidation [28, 29]. Also, decreased activities of the antioxidant enzymes have been observed in the stomach of rats treated with glucocorticoids, and exposure to physiological levels of these hormones exacerbates reactive oxygen species generation [30]. Previous studies in our laboratory have demonstrated the role and involvement of reactive oxygen species in the pathogenesis of acute restraint stress-induced disturbances, and that protection against the reactive oxygen species induced by these stressors can ameliorate gastric injury [8, 13].

Immobilization stress is followed by an increase in free radical levels, especially in lipid peroxidation in plasma and many tissues [8, 13, 28, 29]. Moreover, a changed activity of the antioxidant enzymes Cu/Zn superoxide dismutase and glutathione peroxidase in various tissues of rats with glucocorticoids has also been observed. Stress may thus impair antioxidant defenses, leading to oxidative damage, considerably changing the balance between oxidative and antioxidative factors in stomach [31, 32]. The antioxidant defense system includes enzymes, such as SOD, which converts superoxide radicals into H_2O_2 and catalase, which detoxificates of H_2O_2. As seen in the present study, the increase of SOD activity may cause to convert the superoxide radicals into H_2O_2 and catalase detoxificates of H_2O_2. So gastric mucosal destruction resulting from chronic restraint stress may be less than that of acute stress. It is important to point out that after exposure to chronic restraint stress, glucocorticoid release by the stress situation is decreased when compared to the first

exposure to this type of stress [33]. Repeated exposure to stress leads to a process of adaptation to that stimulus. Therefore the animals exposed repeatedly to restraint stress could be caused to different physiological and behavioral responses, and the changes observed in the present study possibly represent adaptation to this chronic situation, such as the increased activity of SOD. This evidence could imply that exposure to repeated stress induces physiological processes. It has been shown that repeated exposure to stress has detrimental effects on several cell functions in many species and various tissues. In a previous study [8], we demonstrated that a single exposure to restraint stress caused to excessively lipid peroxidation and severe damage in gastric mucosa. The excessive production of reactive oxygen substances and low antioxidant capacity may cause severe damage in gastric mucosa of rats exposed to acute stress. Therefore we conclude that the index of repeated stress-induced gastric lesion was less than that of acute stress. This situation could be due to the increase of SOD activity in gastric mucosa as observed in our study. SOD catalyzes the dismutation of superoxide radical anion into less noxious hydrogen peroxide (H_2O_2), which is further degraded by catalase or glutathione peroxidase. Catalase is an enzyme that accelerates the degradation of H_2O_2 into water and oxygen. The second pathway of H_2O_2 metabolism depends on the activity of glutathione peroxidase and cooperating glutathione reductase. The reduction of H_2O_2 into water by glutathione peroxidase is accompanied by the conversion of glutathione from reduced form into oxidized form [34]. SOD activity reflects the antioxidative properties of various tissues, including gastric mucosa [35, 36]. We determined that repeated restraint stress caused an increase in the SOD activity, and that SOD activity was unchanged in rats exposed to single restraint stress.

In this study, we determined the effect of repeated stress on lipid peroxidation and gastric damage in rats. The results of the present study demonstrated that L-carnitine attenuated repeated stress-induced gastric mucosal injury and significantly inhibited the increase in TBARS production, an index of lipid peroxidation in gastric tissue. It has been reported that two different effects of L-carnitine may be distinguished. The scavenger effect toward oxygen reactive species has been demonstrated by Ronca *et al.* [37]. L-Carnitine inhibits hydroxyl radical production in the Fenton reaction system. On the other hand, the preventive effect of L-carnitine on the formation of oxygen reactive species because of the xanthine/xanthine oxidase system has been shown by Di-Giacomo *et al.* [16]. The reducing effect of L-carnitine on gastric damage could be related to the increasing effect on catalase activity in stressed rats. Pretreatment with carnitine did not change SOD activity, but it increased catalase activity in stressed rats.

Physical stress has been shown to induce significant gastric mucosal injury in rats. It has been demonstrated that water immersion and restraint stress results in an increase in gastric epithelial cell loss and macroscopic mucosal injury [10]. Several mechanisms are believed to be important in protecting the gastric and duodenal mucosa from damage by stressors. These defense mechanisms include mucus content, mucosal blood flow, cell renewal, and bicarbonate secretion. Previous studies demonstrated that restraint stress reduces PGI_2 and PGE_2 production in gastric mucosa [38, 39]. PGs play important roles in the regulation of gastric mucosal integrity. A reduction of these compounds leads to decreases in mucus synthesis and mucosal blood flow resulting in the susceptibility of gastric mucosa to gastric acid and noxious factors [40]. The stress-induced depletion of gastric wall mucus was prevented by L-carnitine. It implies that a concomitant increase in prostaglandins and mucus level contributes to protect the stomach from stress injury. A copious amount of gastric mucus is secreted during superficial mucosal damage and provides a favorable microenvironment in repair by restitution [41]. The protective effect of L-carnitine against repeated stress may be related to prevent stress-induced reduction in prostaglandin and mucus content of gastric mucosa.

The experimental data stated that the generation of gastric ulcer caused by chronic stress was less than that of acute stress compared with our previous study [8]. Pretreatment of rats with L-carnitine could partly protect the gastric mucosa against the harmful effect of chronic stress. The data presented herein suggest that the antioxidant effect and the increasing effect on PGE_2 production of L-carnitine in gastric mucosa is relevant for its enhanced mucosal resistance to chronic restraint stress.

This work was supported by the Research Foundation of Akdeniz University (Project number: 99.01.0103.05).

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