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ORIGINAL PAPER

Insulin like growth factor-1 (IGF-1) decreases ischemia-reperfusion induced apoptosis and necrosis in diabetic rats

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Abstract Previous evidence supports the view that insulin, as well as insulin like growth factor-1 (IGF-1) provides neurotropic support for neurons in the central nervous system (CNS) and peripheral nervous system (PNS). In the present study we evaluated the effects of the intravenous infusion of IGF-1 on both necrosis and apoptosis in the CNS of streptozotocin induced diabetic animals before and/or following middle cerebral artery occlusion (MCAO) with reperfusion. The lesion volume was used as an index of necrosis and the sensorimotor cortex (layers 5 and 6) as well as the CA1 and CA3 regions of the hippocampus were analyzed for apoptosis using TUNEL staining and Caspase-3 immunoreactivity. A large lesion volume was produced in diabetic animals after 2-h MCAO and 24-h reperfusion. Diabetic animals also had an elevated basal level of apoptotic cells that are bilaterally distributed. Apoptosis was further increased over basal after 2-h MCAO and 24-h reperfusion. The acute administration of IGF-1 30-min before or 2 h after MCAO followed by 24-h reperfusion decreased the lesion volume as well as the number of apoptotic cells in the cortical penumbra. Apoptosis as assessed by TUNEL and caspase-3 immunoreactivity was decreased in select sensorimotor cortex and hippocampal areas.

We conclude that treatment with IGF-1 before or after ischemic insult significantly decreases both lesion volume and apoptosis in selected areas of the cortex and hippocampus.

Keywords Diabetes · Ischemia-reperfusion · Insulin like growth factor-1 (IGF-1)

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Introduction

Insulin like growth factor-I (IGF-1) is a multifunctional polypeptide growth factor secreted by the liver and other tissues in response to stimulation by growth hormone. Among its general biological actions, IGF-1 promotes both cell proliferation and differentiation. Subsequent to ischemic brain injury, IGF-1 as well as the IGF-1 receptor and the IGF-1 binding proteins levels are increased in the infarct zone [1]. Moreover, these proteins were differentially expressed in different brain cell populations. For example, IGF-1 mRNA was abundant in the affected astrocytes suggesting that IGF-1 plays a role in astrocyte's metabolic response to ischemic insult [1]. At physiological concentrations both insulin and IGF-1 enhanced neurite outgrowth in cultured embryonic sensory, sympathetic, and motor neurons [2]. IGF-1 has also been shown to act on the vascular endothelium to increase flow as well as constitutive nitric oxide synthase activity via an Akt-catalyzed phosphorylation [3, 4]. This response leads both to an overall increase in blood flow, NO synthesis and reverses the effects noted in growth hormone deficiency [4, 5]. Additionally, IGF-1 has been shown to prevent apoptosis of vascular smooth muscle via an NO mediated mechanisms [6, 7].

Diabetes has been demonstrated to affect both the peripheral nervous system (PNS) and central nervous system (CNS) by increasing apoptotic events [8, 9]. Apoptosis associated with diabetes has also been demonstrated in various tissues such as the retina, cardiovascular system and PNS [9–11]. Several metabolic mechanisms of diabetes-induced apoptosis have been proposed. For example, altered nerve polyol metabolism, non-enzymatic glycosylation, altered NA^+/K^+-ATPase activity, depletion of myo-inositol, and impairments in both neural NA^+/K^+-ATPase and nitric oxide activities have been implicated in the progressive nerve cell loss observed in diabetes [9–13]. We have previously demonstrated an increase in apoptosis in the CNS of diabetic animals [8, 14]. Also, we have demonstrated that middle cerebral artery occlusion (MCAO) with reperfusion exacerbated the CNS lesion in diabetic rats [14].

Since cerebral ischemia (stroke) is a major risk factor in diabetes, in the present study our aim was to characterize the effects of acute intravenous administration of IGF-1 before MCAO or after MCAO in diabetic animals following 24-h reperfusion. CNS ischemic-lesion volume as well as the spatial distribution of apoptic cells in streptozotocin-induced diabetic male Wistar rats were evaluated in layers 5 and 6 of the sensorimotor cortex and the CA1 and CA3 regions of the hippocampus (penumbral area) ipsilateral to the MCAO.

Results

Physiological parameters

Streptozotocin-induced diabetic rats had increased non-fasting blood glucose, 502 ± 8 mg/dl compared to normal controls 178 ± 5 mg/dl. Acute treatment with IGF-1 before MCAO resulted in a significant decreased blood glucose to 358 ± 27 mg/dl for 2 h following MCAO (Table 1). Also, insulin levels in the diabetic animals were significantly lower compared to those in normal controls. IGF-1 treatment before or after MCAO did not significantly alter insulin levels in diabetic animals.

Infarct volume

Infarct volume was determined using the TTC stain and used as an index of cell death (necrosis). Diabetic animals subjected to a 2-h MCAO/24-h reperfusion had a lesion volume significantly greater than that of the non-diabetic counterparts (31.5 ± 2.5% and 3.02 ± 2.36%, respectively). Acute treatment with IGF-1, 30-min before MCAO, significantly decreased the size of the lesion volume (Lane C vs. Lane D; Figs. 1, 2). Treatment with IGF-1 2 h after MCAO also decreased the size of the lesion, but not significantly (Lane C vs. Lane D; Figs. 1, 2).

Fig. 1 Triphenyltetrazoium chloride (TTC) stained consecutive sections (most rostral at top) for lesioned white area in (**A**) non-diabetic normal MCAO and 24-h reperfusion; (**B**) acute IGF-1 treatment 2 h following MCAO and 24-h reperfusion in diabetic rats; (**C**) acute IGF-1 treatment 30-min before 2-h MCAO/24-h reperfusion in diabetic rats; and (**D**) no treatment 2-h MCAO/24-h reperfusion in diabetic rats

Table 1 Blood glucose and insulin values in non-fasted male Wistar rats following 2-h MCAO/24-h reperfusion in: (A) non-diabetic normal, (B) no treatment control diabetic, (C) IGF-1 treatment 30-min before 2-h MCAO and (D) IGF-1 treatment after 2-h MCAO

	A	B	C	D
Glucose (mg/dl)	178 ± 4.94	502 ± 8.86*	358 ± 27*#	400 ± 35*
Insulin (ρmol/ml)	0.76 ± 0.084	0.31 ± 0.03*	0.28 ± 0.02*	0.29 ± 0.06*

Note: The values represent the mean ± SEM

* $P < 0.05$ versus normal

# $P < 0.05$ versus diabetic no treatment, one way ANOVA

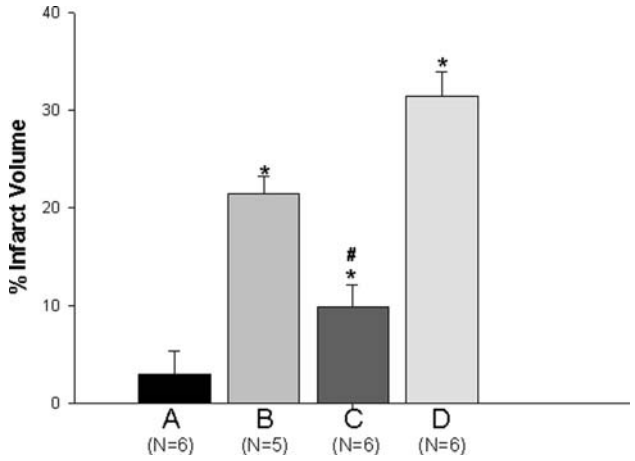

Fig. 2 Percent infarct volume in normal and diabetic animals with 2-h MCAO followed by 24-h reperfusion. (**A**) non-diabetic normal MCAO and 24-h reperfusion; (**B**) diabetic IGF-1 treatment after 2-h MCAO; (**C**) diabetic IGF-1 treatment 30 min before MCAO; (**D**) diabetic control

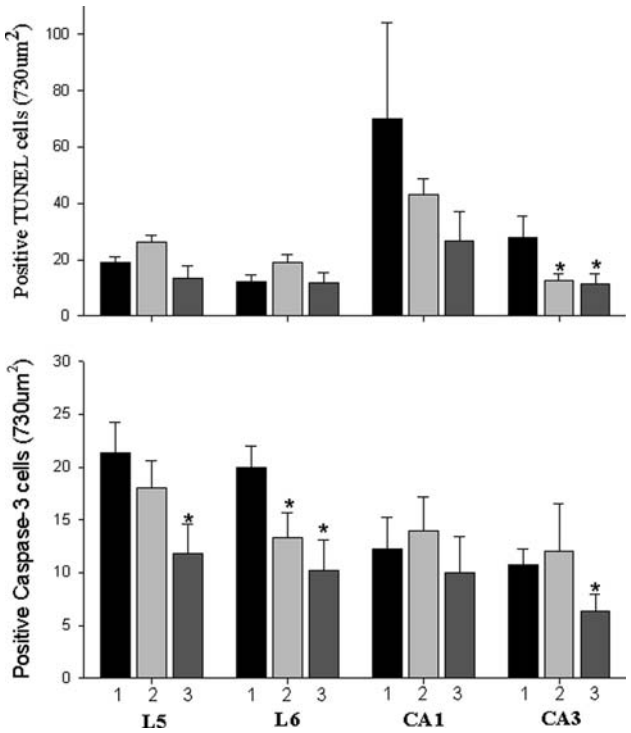

Fig. 3 Number of TUNEL and caspase-3 positive cells in the sensorimotor area of cerebral cortex (layer-5 (L5) and layer-6 (L6)) and CA1 and CA3 regions of the hippocampus in: (**1**) diabetic reperfused (D/R, $N = 3$); (**2**) D/R treated with IGF-1 before ($N = 3$); and (**3**) D/R treated with IGF-1 ($N = 3$) after 2-h/24-h reperfusion MCAO

TUNEL and caspase-3 activity in diabetic animals

Representative histological sections were evaluated for apoptosis using TUNEL staining and caspase-3 activity in diabetic animals subjected to a 2-h MCAO followed by 24-h reperfusion. It can be observed in Fig. 3 that acute treatment with IGF-1, either before or after MCAO significantly decreases TUNEL staining in the CA3 region of the hippocampus. The decrease in the elevated CA1 region did not achieve significance. IGF-1 treatment before or after MCAO significantly decreased caspase-3 immunostaining in L5 and L6 of the cerebral cortex as well as the CA3 region of the hippocampus (Fig. 3). The histological appearance of these areas for both TUNEL and caspase-3 staining can be seen in Figs. 4 and 5.

Discussion

Apoptosis has been reported in most of the chronic complications of diabetes such as pancreatic beta-cell dysfunction, diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy [11, 15, 16]. Previously we reported that a 2-h MCAO followed by a 24-h reperfusion period exacerbated both necrosis and apoptosis in a type-1 diabetic animal model [8, 14]. Infarct volume in diabetic animals following reperfusion was identical to that of diabetic animals subjected to 24-h MCAO, in both cases including large areas of the ipsilateral cortex and the rostral striatum. Once the ischemic insult was initiated in diabetic animals, restoring blood flow to these animals caused unexpectedly as severe a lesion as that produced by the 24-h MCAO without reperfusion [8, 14]. Conversely, restoring blood flow in non-diabetic animals prevented the formation of the large infarct size seen with the longer ischemic period [8].

Our study confirms that diabetic animals had a significantly greater lesion volume subsequent to the 2-h MCAO/24-h reperfusion when compared to their non-diabetic counterpart. This suggested a greater vulnerability of cells in the penumbral area and thus leads to the formation of a larger area of necrosis in the diabetic animals. We also clearly show that acute IGF-1 treatment before ischemic insult significantly decreased the lesion volume following restoration of blood flow. Additionally, this acute IGF-1 treatment appeared to significantly ameliorate the subsequent (24 h later) ischemia/reperfusion induced neuronal apoptosis. We detected the actual significant decrease in apoptotic cell numbers in only selected locations. Our observation is supported by the fact that insulin has been demonstrated to improve cell survival in different diabetic models [17, 18]. Insulin like growth factor-1 (IGF-1) is naturally occurring in the brain and also IGF-1 plays an important role in CNS development, survival as well as differentiation of both neuronal and glial cells [19]. Since the neuronal apoptosis that we observed was not affected as profoundly as necrosis-induced lesion volume subsequent

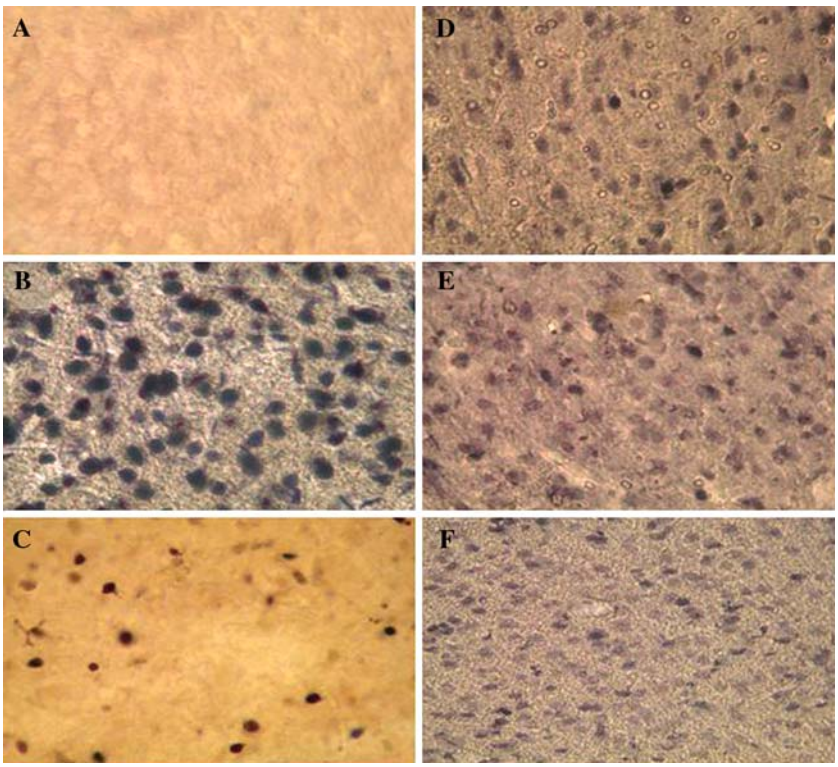

Fig. 4 TUNEL positive cells in layer-5 of sensorimotor cortex of type-1 diabetic animals. (**A**) negative control; (**B**) positive control; (**C**) no treatment; (**D**) acute IGF-1 treatment 2 h following MCAO/24-h reperfusion; (**E**): acute IGF-1 treatment 30-min before 2-h MCAO/24-h reperfusion; and (**F**): 2-h MACO/24-h reperfusion, no treatment

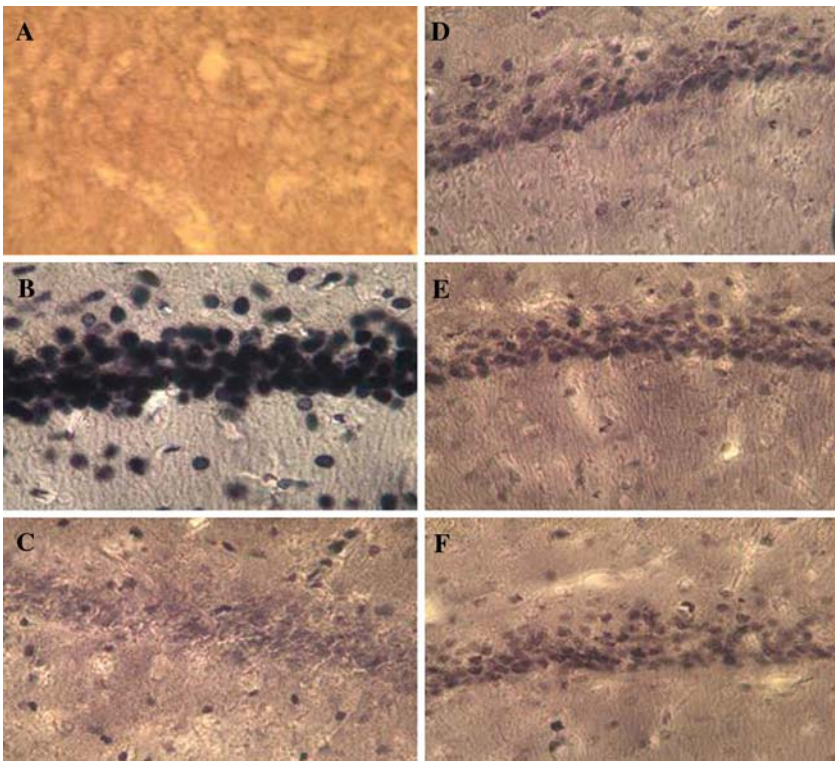

Fig. 5 TUNEL positive cells in CA1 region of hippocampus of type-1 diabetic animals. (**A**) negative control; (**B**) positive control; (**C**) no treatment; (**D**) acute IGF-1 treatment 2 h following MCAO/24-h reperfusion; (**E**) acute IGF-1 treatment 30-min before 2-h MCAO/24-h reperfusion; (**F**) 2-h MCAO/24-h reperfusion, no treatment

to IGF-1 treatment, we propose that a primary beneficial effects of IGF-1 on ischemia/reperfusion is to prevent neurons from progressing to apoptosis or the development of vascular-mediated cerebral edema [11, 20]. This is also supported by the observation that the administration of IGF-1 before or after spinal cord injury was neuroprotective

via attenuating disturbances of the fluid microenvironment of the spinal cord [21].

Our histological data further demonstrated that the CA1 region to be very sensitive to ischemia and is a resistant area of the hippocampus after acute IGF-1 treatment. Different regions of the hippocampus have selective vulnerabilities to hypoxemia and ischemia [22]. Pyramidal neurons in the CA1 and CA3 regions of the hippocampus and those in layers III and V of the cerebral cortex are also selectively vulnerable to death after injury by ischemia and reperfusion [23]. Ultrastructural evidence have demonstrated that most of the damage associated with reperfusion in vulnerable neurons involves disaggregation of polyribosomes, peroxidative damage to unsaturated fatty acids in the plasma membrane, and distinct alterations in the structure of the Golgi apparatus that is responsible for membrane assembly [24]. In addition, as a result of reperfusion, inhibition of protein synthesis, involving alteration of the translation initiation factors, specifically serine phosphorylation of the alpha subunit of eukaryotic initiation factor-2 (eIF-2 alpha), result in death of the selectively vulnerable neurons [23]. IGF-1 may be important in blocking this series of events.

In summary, in this study we have further confirmed that diabetic animals have increased levels of apoptosis in the CNS, and that restoration of blood flow after ischemia exacerbates both necrosis and neuronal apoptosis in the CA1 and CA3 regions of the hippocampus as well as neuronal necrosis in cerebral cortex ipsilateral to MCAO. Our data further suggests that the acute administration of IGF-1 ameliorates both infarct volume and apoptosis by possibly preventing directly initiation of apoptosis in neurons or in glia or vascular cells by preventing secondary cell damage from reperfusion.

Materials and Methods

Animals

Male Wistar rats (280–300 g; Harlan, Indianapolis, IN) were used and housed two to a cage in a temperature-controlled environment (23°C) with an alternating, 12-h light–dark cycle. The rats were given rodent chow and water ad libitum. All animal care and surgical procedure were performed in accordance with guidelines approved by the National Institutes of Health and the Wayne State University Animal Investigation Committee. Diabetes was induced in 8-week old normal rats by a single intravenous tail vein injection of streptozotocin (STZ) (50 mg/kg dissolved in sodium citrate, 0.1 mM, pH 4.5). A blood sample was collected 7-days after STZ injection, and plasma glucose was determined using a glucose analyzer (Yellow Springs Instruments Co., Yellow Springs, OH). Diabetes was defined by a blood glucose >300 mg/dl. Animals were used 4–6 weeks later without insulin supplements.

Before the animals were subjected to middle cerebral artery occlusion (MCAO), a blood sample was collected from the orbital sinus for determination of glucose, insulin (ICN Pharmaceuticals, Orangeburg, NY) and C-peptide (Linco Research, St Charles, MO) concentrations.

Middle cerebral artery occlusion and reperfusion

The experimental MCAO rat model was conducted as described previously [25]. Animals were anesthetized and maintained with 1–3% halothane in 30% oxygen with a facemask. Rats were placed in the supine position on a heated pad, with body temperature maintained at 37 ± 0.5°C using a rectal thermometer. Under an operating microscope, the right common carotid artery including its bifurcation was exposed and dissected. All branches of the external carotid artery were isolated, coagulated and transected. The internal carotid artery was then isolated and clamped with a small vascular clip. The common carotid artery was also clamped with a vascular clip. The stump of the external carotid artery was cut and a nylon filament thread (SP 184, 4–0) was inserted into the right external carotid artery and carefully maneuvered into the internal carotid artery. The filament was then passed up the lumen of the internal carotid artery ultimately blocking the middle cerebral artery (MCA) at its origin. The distance from the bifurcation of the common carotid artery to the distance of the suture was ~18.5 mm in all rats. A successful occlusion of the right MCA was achieved when the left forelimb was paretic after nylon filament block [26].

IGF-1 infusion

The femoral vein was isolated and cannulated. One group of diabetic animals was infused intravenously with IGF-1 (Tercica Inc., Brisbane, CA) (5 mg/kg) 2-h before the MCA was put in place. Another group of diabetic animals was infused intravenously with IGF-1 (5 mg/kg) 2 h following the MCA occlusion. The reperfused animals were reanesthetized 2 h after MCAO, and reperfusion was established by the withdrawal of the nylon filament. Animals were reperfused for 24-h before they were sacrificed.

Histological assessments

Rats subjected to 2-h MCAO followed by 24-h reperfusion, were anesthetized with pentobarbital (50 mg/kg body wt., intraperitoneally) and sacrificed by cardiac perfusion of

saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffered solution (PBS, pH 7.5). Subsequent to perfusion, the brains were carefully removed and stored in the perfusion fixation solution at 4°C for a minimum of 24 h. The brains were then sectioned coronally (50-μm) using a vibratome. Adjacent brain sections from single brains were either processed for activated caspase-3 immunohistochemistry or TUNEL positive staining.

TUNEL Staining: The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay was utilized on brain sections using the In Situ Cell Death Detection Kit POD (Roche; Indianapolis, IN). A dark brown color indicating DNA breaks developed after incubation with DAB (3-3′-diamonobenzidine tetrachloride) and hydrogen peroxide. Positive TUNEL stained cells were counted in a 730-μm2 area of the cortical penumbra using a microscope provided with a graded eyepiece reticule at a magnification of 240×.

Activated caspase-3 Assay: To determine caspase-3 immunoreactivity, brain sections were washed three times in 0.1 M PBS (pH 7.5), treated with 3% hydrogen peroxide to quench endogenous peroxidases, washed again 3× with PBS, and then treated with 0.1% triton X 100 for 15 min. Sections were incubated with normal blocking solution (ABC kit Vector Laboratories, Burlingame, CA) for 30 min and then incubated with a rabbit polyclonal antibody generated against activated caspase-3 (caspase-3 Sigma, St Louis) at a concentration 1:200 at 4°C overnight. The next day, samples were incubated in secondary antibody for 1-h and then incubated for 30-min using elite ABC reagent (ABC kit Vector Laboratories, Burlingame CA). Finally, the sections were incubated in a peroxidase substrate kit DAB for 10-min, and mounted on slides.

Infarct Volume: Rats subjected to 2-h MCAO followed by 24-h reperfusion, were anesthetized with pentobarbital (50 mg/kg body wt, intraperitoneally) and decapitated. Brains were immediately removed and placed in ice-cold saline for 5 min. Each brain was then placed in a brain matrix and coronal sections were cut into 2-mm slices. Brain slices were immediately immersed in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) solution (in saline) at 37°C for 30-min, followed by 4% paraformaldehyde solution. Triphenyltetrazolium chloride (TTC) staining was used for lesion volume (index of necrosis) determination [27]. Ischemic volume was determined from adjacent consecutive sections by using the Metamorph software to outline the ischemic areas and total area factoring the total area into volume and expressing ischemic volume as a percentage of the whole brain.

Statistical analysis

A one-way post hoc ANOVA test was used to compare data between groups and Student *t*-test was used to compare the results between two groups.

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