

## The Role of Insulin in Norepinephrine Turnover and Thermogenesis in Brown Adipose Tissue after Acute Cold-Exposure

KEIJI YOSHIOKA, TOSHIHIDE YOSHIDA, YASUO WAKABAYASHI\*,  
HITOSHI NISHIOKA AND MOTOHARU KONDO

*First Department of Internal Medicine and Biochemistry\*,  
Kyoto Prefectural University of Medicine,  
Kamikyo-ku, Kyoto 602, Japan*

### Abstract

The role of insulin in norepinephrine turnover (NE) and thermogenesis in brown adipose tissue (BAT) after acute cold-exposure was studied using streptozocin (STZ)-induced diabetic rats. NE turnover was estimated by the NE synthesis inhibition technique with  $\alpha$ -methyl-p-tyrosine. BAT thermogenesis was estimated by measuring mitochondrial guanosine-5'-diphosphate (GDP), cytochrome oxidase activity and mitochondrial oxygen consumption in BAT at an ambient temperature of 22°C and during a six-hour cold-exposure at 4°C. In insulin-deficient diabetic rats, the NE turnover, mitochondrial GDP binding, cytochrome oxidase activity and mitochondrial oxygen consumption in BAT at 22°C were significantly reduced, compared with those of control rats. Treatment of STZ-induced diabetic rats with insulin prevented a decrease in NE turnover and BAT thermogenesis. Acute cold-exposure increased the NE turnover of BAT in insulin-deficient diabetic rats. The BAT thermogenic response to acute cold-exposure, however, did not occur in insulin-deficient diabetic rats. These results suggest that insulin is not essential in potentiating NE turnover in BAT after acute cold-exposure, but is required for cold-induced thermogenesis.

Brown adipose tissue (BAT) is now recognized as being a main effector of cold-induced thermogenesis (Foster and Frydman, 1979) and diet-induced thermogenesis (Rothwell and Stock, 1979).

Thermogenesis in BAT is regulated primarily by the sympathetic nervous system (SNS) (Landsberg and Young, 1984; Giarardier and Seydoux, 1986).

Insulin-deficient diabetes is known to impair thermogenesis in response to diet (Rothwell and Stock, 1981). The decreased BAT thermogenesis in diabetic animals could be partly attributed to the reduced SNS activity in BAT (Yoshida *et al.*, 1985, 1987). It may also be due to a defect

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Address all correspondence to KEIJI YOSHIOKA, First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kamikyo-ku, Kyoto 602, Japan

in mitochondrial  $\beta$ -oxidation and changes in abundance (Seydoux *et al.*, 1982, 1984) or guanosine-5'-diphosphate (GDP) binding properties (Jamal and Saggerson, 1988) of uncoupling proteins in the BAT of diabetic animals.

Diabetic animals are also intolerant to cold-acclimation (Himms-Hagen, 1986). A recent report, however, has shown that a thermogenic response to acute cold-exposure was activated in 2-day diabetic rats by measuring BAT temperature and cytochrome oxidase activity in the BAT (Shibata *et al.*, 1987). Up to date, however, SNS activity or biochemical changes in the BAT mitochondria after acute cold-exposure are less substantial in long-term insulin-deficient diabetic animals.

Therefore, the aim of this study is primarily to investigate whether SNS activity is increased by acute cold-exposure in an insulin-deficient state, by measuring norepinephrine (NE) turnover, and secondly to elucidate whether acute cold-induced thermogenesis occurs in an insulin-deficient state, by measuring GDP binding, cytochrome oxidase activity and oxygen consumption in the BAT mitochondria.

## Materials and Methods

Two hundred and four female Sprague-Dawley rats (weighing, ca. 200 g, 8 w) were purchased from Charles River Japan (Osaka, Japan). One hundred and fifty rats were injected intravenously with streptozocin (STZ) (Upjohn, Kalamazoo, Michigan, USA) at a dose of 65 mg/kg freshly dissolved in ice-cold citrate buffer (pH 4.5), and the other 54 rats with physiological saline (Control group). The animals were then housed in wire-mesh cages at a constant temperature of  $22 \pm 2^\circ\text{C}$  with artificial light from 0600 h to 1800 h. They were allowed free access to commercial powdered chow (Charles River Japan) and tap water. The diabetic condition was verified by measuring plasma glucose levels with an auto-analyzer (Beckman, Fullerton, California, USA) 7 days after the injection of STZ. One hundred

and forty-four diabetic animals having a plasma glucose level greater than 400 mg/dl were used in this experiment. One week after the induction of diabetes, 54 STZ-diabetic rats were treated with NPH insulin (Nordisk, Gentofte, Denmark) at a dose of 4–8 U every evening (1800 h) [STZ (INS) group], and the other 90 STZ-diabetic rats were untreated [STZ (untreated) group]. Plasma glucose levels were measured at 1700 h once a week, and the dose of insulin was adjusted as followed: NPH insulin was given at a dose of 8 U for plasma glucose levels  $> 500$  mg/dl, 6 U for 500–400 mg/dl, and 4 U for 400–300 mg/dl. Four weeks after STZ administration, the body weight, food intake and plasma glucose levels were measured. Two days after the measurement of these parameters, the following experiments were carried out.

### *Experiment 1: Norepinephrine (NE) turnover*

Thirty rats each from the STZ(untreated), STZ(INS) and control groups were used in the experiment on NE turnover. NE turnover in interscapular BAT (IBAT) in each group was measured at an ambient temperature of  $22^\circ\text{C}$  and after a six-hour cold-exposure at  $4^\circ\text{C}$ . The experiment started at 0900 h. After the intraperitoneal injection of the methyl ester of  $\alpha$ -methyl-p-tyrosine (80 mg/kg, Sigma, St. Louis, MO, USA) dissolved in saline, 12 rats each in the STZ(untreated), STZ(INS) and control groups were placed in a cold room ( $4^\circ\text{C}$ ), and 12 rats each in the STZ(untreated), STZ(INS) and control groups were kept at an ambient temperature of  $22^\circ\text{C}$ . Six rats each in the STZ(untreated), STZ(INS) and control groups were not injected and served as the 0 h reference. Half of the 24 animals in each group were killed at 3 h and the other half killed at 6 h. IBAT was rapidly removed, dissected from the connective tissue, weighed, homogenized in ice-cold 0.1 N perchloric acid containing 0.1 mM reduced glutathione in a polytron (Kinematica, Lucerne, Switzerland) and centrifuged at  $0^\circ\text{C}$ . Aliquots of the supernatant were stored at  $-70^\circ\text{C}$  for later determination of NE (usually within 1 week). NE was assayed radioenzymatically with a minor modification (Yoshida *et al.*, 1983) of the method of Peuler and Johnson (1977). All data are presented as the mean  $\pm$  S.E. Statistical analysis was performed by the analysis of variance and covariance (Zar, 1974). Comparisons of fractional turnover rates were

calculated as the product of the fractional turnover rate (k) times the endogenous NE concentration at point zero. 95% confidence intervals were determined for the NE turnover rates as described previously (Taubin *et al.*, 1979).

### Experiment 2: Brown adipose tissue thermogenesis

Twenty-four rats each in the STZ(INS) and control groups, and 60 rats in the STZ(untreated) group were used in the experiment on BAT thermogenesis. Mitochondrial GDP binding, cytochrome oxidase activity and oxygen consumption in the IBAT of each group were measured at 22°C and a six hour cold-exposure at 4°C. The rats were killed by cervical dislocation at 0900 h. Interscapular BAT was rapidly removed and dissected from the connective tissue. For one measurement of the parameters described below, interscapular BAT samples were collected from 3 rats in the control and STZ(INS) groups, and 6 rats in the STZ(untreated) groups at 22°C, and from one rat in the control and STZ(INS) groups, and 4 rats in the STZ(untreated) group at 4°C. BAT samples were weighed and homogenized in an ice-cold medium (pH 7.2) containing 250 mM sucrose and 5 mM TES. Portions of the homogenates were used for the measurement of cytochrome oxidase activity. The mitochondria were isolated by differential centrifugation according to the procedure described by Cannon and Lindberg (1973). The tissue protein and mitochondrial protein were estimated by the method of Lowry *et al.* (1951). Mitochondrial GDP binding

was determined by incubation and filtration according to a minor modification (Yoshioka *et al.*, 1988) of the method of Nicholls (1976). The mitochondria were incubated at 20°C in 0.5 ml of medium containing [<sup>3</sup>H]-GDP (1.30  $\mu$ Ci), [<sup>14</sup>C]-sucrose (0.123  $\mu$ Ci), 100  $\mu$ M sucrose, 100  $\mu$ M potassium atractyloside, 20 mM TES (pH 7.1), 10 mM choline chloride and 5  $\mu$ M rotenone. After 7 minutes of incubation, 0.4 ml aliquots containing 0.26 mg of mitochondrial protein were withdrawn and filtered through a nitrocellulose membrane filter (Sartorius, Göttingen, W. Germany, pore size 0.45  $\mu$ m). The filters were counted for [<sup>3</sup>H] and [<sup>14</sup>C] in a scintillation counter (Packard, Downers, Grove, IL, USA). Cytochrome oxidase activity was measured spectrophotometrically with a Double-Beam Spectrophotometer (UV-140-02, Shimadzu, Kyoto) at 25°C in 1 ml of a medium consisting of 100 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA and 30  $\mu$ M reduced cytochrome c after treating the homogenates with 1% Lubrol by the method of Yonetani and Ray (1965). Mitochondrial respiration was determined polarographically in 2 ml of medium consisting of 100 mM KCl, 20 mM TES (pH 7.2), 4 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 4  $\mu$ M rotenone and 10% defatted BSA at 25°C with Rank oxygen equipment (Rank Bros, Cambridge, UK). Mitochondrial protein (850  $\mu$ g) was added to a medium and 10 mM  $\alpha$ -glycerophosphate was added as a substrate for mitochondrial respiration. All data are presented as the mean  $\pm$  S.E. Statistical analysis was done by Student's *t*-test.

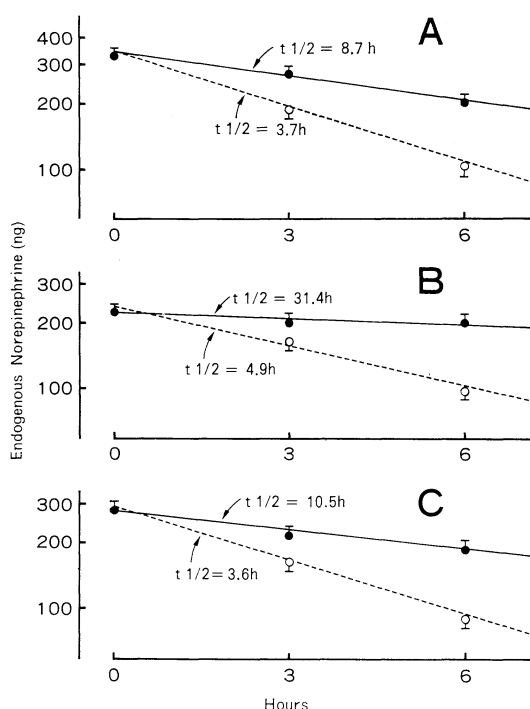
**Table 1.** Body weight, food intake, plasma glucose level and pancreatic insulin content in control, STZ(untreated) and STZ(INS) rats at an ambient temperature of 22°C.

	Control	STZ(untreated)	STZ(INS)
n	18	36	18
Body weight (g)	258.3 $\pm$ 5.5	189.3 $\pm$ 6.2 <sup>b</sup>	245.2 $\pm$ 6.0 <sup>e</sup>
Food intake (g/day)	24.0 $\pm$ 11.4	45.7 $\pm$ 5.8 <sup>a</sup>	31.1 $\pm$ 5.2
Plasma glucose level (mg/dl)	89.9 $\pm$ 4.4	462.2 $\pm$ 17.2 <sup>b</sup>	186.2 $\pm$ 17.9 <sup>c,e</sup>
Pancreatic insulin content (mU/g $\cdot$ pancreas)	1018.0 $\pm$ 52.6	24.1 $\pm$ 10.9 <sup>b</sup>	25.2 $\pm$ 13.4 <sup>d</sup>
Values are shown as the mean $\pm$ S.E. n: Number of animals in each group			
Control vs STZ(untreated)	: a; p < 0.05		
	: b; p < 0.001		
Control vs STZ(INS)	: c; p < 0.02		
	: d; p < 0.001		
STZ(untreated) vs STZ(INS)	: e; p < 0.001		

## Results

Table 1 shows body weight, food intake, plasma glucose level and pancreatic insulin content of the control, untreated STZ [STZ (untreated)] and STZ treated with insulin [STZ(INS)] groups 4 weeks after the STZ administration. The STZ(untreated) group was significantly lighter in body weight than the control or STZ(INS) groups ( $p < 0.001$ ). Food intake was significantly greater in the STZ(untreated) group than in the control group ( $p < 0.05$ ). However, daily insulin injections given to STZ rats decreased food intake to levels equal to the control. Plasma glucose levels in the STZ (untreated) group were markedly higher than those in the control or STZ(INS) groups ( $p < 0.001$ ). Pancreatic insulin content was significantly lower in the STZ (untreated) and STZ(INS) groups than in the control group ( $p < 0.001$ ).

**Experiment 1:** Table 2 and Figure 1 show the NE turnover data for IBAT of the control, STZ(untreated) and STZ(INS)



**Fig. 1.** Effect of ambient temperature (22°C) (●—●) and acute cold-exposure (4°C) (○---○) on the norepinephrine turnover in the interscapular brown adipose tissue of the Control (A), STZ(untreated)(B) and STZ(INS)(C) rats.

**Table 2.** Effect of ambient temperature (22°C) and acute cold-exposure (4°C) on norepinephrine turnover in the interscapular brown adipose tissue of control, STZ(untreated) and STZ(INS) rats.

	Ambient temperature (°C)	IBAT		
		endogenous NE (ng/organ)	k (%/h)	NE·Tr (ng/organ/h)
Control	22	330.2±13.5	7.9±1.1	26.1±4.8
	4		18.7±1.8 <sup>a</sup>	61.7±8.8
STZ (untreated)	22	225.5±11.2 <sup>c</sup>	2.2±1.0 <sup>b</sup>	5.0±2.6
	4		14.1±1.5 <sup>a</sup>	31.8±5.1
STZ (INS)	22	280.5± 8.3 <sup>d,e</sup>	6.6±1.3	14.9±4.0
	4		19.3±1.6 <sup>a</sup>	54.1±6.3

The fractional NE turnover rate (k) is expressed as the mean±S.E.

The NE turnover (NE·Tr) is expressed as the mean with 95% confidence limits.

Six rats were used at each time point to obtain the turnover data.

22°C vs 4°C: a;  $p < 0.01$

Control (22°C) vs STZ (untreated) (22°C) : b;  $p < 0.02$

c;  $p < 0.001$

Control (22°C) vs STZ (INS) (22°C)

d;  $p < 0.02$

STZ(untreated) (22°C) vs STZ(INS) (22°C) : e;  $p < 0.02$

**Table 3.** Interscapular BAT (IBAT) weight, mitochondrial (Mt) protein content in IBAT, mitochondrial GDP binding, cytochrome oxidase activity and mitochondrial oxygen consumption in IBAT of Control, STZ (untreated) and STZ (INS) rats both at an ambient temperature of 22°C and after acute cold-exposure of 4°C

	Ambient temperature (°C)	IBAT weight (g)	Mt. protein content in IBAT (mg)	Mt. GDP binding in IBAT (pmol/mg mito. protein)	Cytochrome oxidase activity in IBAT ( $\mu$ mol/min·mg tissue protein)	Mt. oxygen consumption in IBAT (nmol/min·mg mito. protein)
Control	22 (N=6)	0.31 $\pm$ 0.02	4.10 $\pm$ 0.47	153 $\pm$ 13.3	2.91 $\pm$ 0.16	20.6 $\pm$ 2.0
	4	0.35 $\pm$ 0.04	3.81 $\pm$ 0.15	307 $\pm$ 15.1 <sup>e</sup>	2.52 $\pm$ 0.13	33.6 $\pm$ 2.9 <sup>b</sup>
STZ (untreated)	22 (N=6)	0.10 $\pm$ 0.01 <sup>f</sup>	0.82 $\pm$ 0.12 <sup>e</sup>	105.4 $\pm$ 10.8 <sup>d</sup>	1.50 $\pm$ 0.18 <sup>e</sup>	10.4 $\pm$ 0.9 <sup>f</sup>
	4	0.08 $\pm$ 0.01 <sup>k</sup>	0.71 $\pm$ 0.08 <sup>k</sup>	163.1 $\pm$ 19.0 <sup>a,k</sup>	1.43 $\pm$ 0.13 <sup>k</sup>	9.0 $\pm$ 2.7 <sup>k</sup>
STZ (INS)	22 (N=6)	0.36 $\pm$ 0.03 <sup>i</sup>	3.32 $\pm$ 0.32 <sup>i</sup>	143.9 $\pm$ 10.5 <sup>g</sup>	2.32 $\pm$ 0.14 <sup>b,j</sup>	17.9 $\pm$ 2.3 <sup>h</sup>
	4	0.37 $\pm$ 0.03 <sup>m</sup>	3.21 $\pm$ 0.29 <sup>m</sup>	277.8 $\pm$ 9.4 <sup>b,m</sup>	2.17 $\pm$ 0.16 <sup>l</sup>	25.8 $\pm$ 0.7 <sup>b,m,n</sup>

Values are shown as the mean $\pm$ S.E.

22°C vs 4°C : a ; p<0.05  
b ; p<0.01  
c ; p<0.001  
Control (22°C) vs STZ (untreated) (22°C) : d ; p<0.05  
e ; p<0.01  
f ; p<0.001

N : Number of measurements in each group

STZ (untreated) (22°C) vs STZ (INS) (22°C) : g ; p<0.05  
h ; p<0.01  
i ; p<0.001  
STZ (INS) (22°C) vs Control (22°C) : j ; p<0.05  
Control (4°C) vs STZ (untreated) (4°C) : k ; p<0.001  
STZ (untreated) (4°C) vs STZ (INS) (4°C) : l ; p<0.01  
Control (4°C) vs STZ (INS) (4°C) : m ; p<0.001  
n ; p<0.01

groups at an ambient temperature of 22°C and after acute cold-exposure at 4°C. Endogenous NE levels in the IBATs of the STZ(untreated) group were less than those of the control group at an ambient temperature of 22°C ( $p < 0.001$ ). The fractional NE turnover rate ( $k$ ) and the total NE turnover rate in the IBAT of the STZ(untreated) group at 22°C were significantly slower than in the control group. Treatment of the STZ rats with insulin prevented a decrease in the fractional and total NE turnover rates in the IBAT. When STZ(untreated) rats were exposed to cold at 4°C for 6 hours, the NE turnover rate in the IBAT significantly increased as compared with STZ(untreated) rats maintained at 22°C. This phenomenon was also observed in the control and STZ(INS) groups.

**Experiment 2:** Table 3 shows IBAT weight, the mitochondrial protein content in IBAT, mitochondrial GDP binding, cytochrome oxidase activity and mitochondrial oxygen consumption in IBAT of the control, STZ(untreated) and STZ(INS) groups at an ambient temperature of 22°C and also at 4°C. The IBAT weight and mitochondrial protein content in IBAT of the STZ(untreated) group were significantly decreased as compared with those in the control and STZ(INS) groups at both 22°C and 4°C. Mitochondrial GDP binding, cytochrome oxidase activity and mitochondrial oxygen consumption in the IBAT of the STZ(untreated) group were significantly lower than those in the control group at an ambient temperature of 22°C ( $p < 0.05-0.001$ ). A six-hour cold-exposure of the control and STZ(INS) rats at 4°C resulted in enhanced mitochondrial GDP binding and oxygen consumption in the IBAT as compared with those at the ambient temperature of 22°C ( $p < 0.01-0.001$ ). In STZ(untreated) rats, mitochondrial GDP binding in the IBAT was increased by acute cold-exposure at 4°C ( $p < 0.05$ ). However, the mitochondrial oxygen consumption in the IBAT

did not increase.

## Discussion

The present study demonstrates that NE turnover and thermogenesis in BAT were decreased in insulin-deficient diabetic rats, and that acute cold-exposure could activate NE turnover in BAT but not BAT thermogenesis.

In STZ-induced diabetic rats, the NE turnover at an ambient temperature of 22°C, which is a reliable indicator of SNS activity (Young and Landsberg, 1977; Vander Tuig *et al.*, 1982; Yoshida and Bray, 1983), was markedly reduced as compared with that of the control rats. Treatment of STZ-induced diabetic rats with insulin prevented the reduction of the NE turnover in BAT. The present data are completely in accordance with those obtained in our previous studies (Yoshida *et al.*, 1985, 1987).

Acute cold-exposure at 4°C activated NE turnover in BATs not only in the control and STZ(INS) groups, but also in the STZ(untreated) group. These results suggest that insulin is not required for the activation of NE turnover after acute cold-exposure. It is suggested that the center of cold-induced thermogenesis is the preoptic area and spinal cord (Banet *et al.*, 1978) and that of diet-induced thermogenesis is the ventromedial hypothalamus (VMH) (Bray and York, 1979; Yoshida and Bray, 1984). Signals to the SNS from the environmental temperature and diet would be processed separately in the hypothalamus. Therefore, insulin might not be essential for the activation of NE turnover after acute cold-exposure.

It is known that a proton conductance pathway, closely coupled with respiration across the BAT mitochondrial inner membrane, generates BAT thermogenesis (Trayhurn, *et al.*, 1986). The pathway is regulated by a mitochondrial inner membrane protein

'uncoupling protein', which has a high affinity for purine nucleotide. Here GDP is useful in detecting the presence of uncoupling protein (Nicholls, 1976).

The levels of GDP binding, cytochrome oxidase activity and oxygen consumption in BAT mitochondria at 22°C were significantly decreased in the STZ-induced diabetic rats. Treating the STZ-induced diabetic rats with insulin restored these parameters. The present results are in accordance with other reports (Seydoux *et al.*, 1984; Jamal and Saggerson, 1988), in which a decrease in the mitochondrial abundance or reduced GDP binding in BAT contributes to the reduced BAT thermogenesis in insulin-deficient diabetic rats.

The defect in the BAT thermogenesis of insulin-deficient diabetic animals has also been attributed to a marked reduction of the  $\beta$ -oxidation capacity of free fatty acids (Seydoux *et al.*, 1984) as well as to a decreased ability to synthesize or accumulate fatty acids for fuel in BAT thermogenesis (Trayhurn, 1981). Furthermore, STZ-induced diabetic rats may be in the hypothyroid state which may also contribute to the impairment of BAT thermogenesis (Ortiz-Caro *et al.*, 1984; Bestetti *et al.* 1987).

With the activation of NE turnover, acute cold-exposure increased GDP binding in insulin-deficient diabetic rats as well as control rats. Mitochondrial oxygen consumption, however, could not be increased by acute cold-exposure in insulin-deficient diabetic rats. In contrast, acute cold-exposure increased mitochondrial oxygen consumption in the BAT in the control and STZ(INS) groups. The discrepancy between GDP binding and oxygen consumption in BAT mitochondria in insulin-deficient diabetic rats may be due to the 'unmasking' of GDP binding sites of uncoupling protein (Swick and Swick, 1986; Gribskov *et al.*, 1986).

A recent report, however, has shown that acute cold-exposure increases heat

production of IBAT in 2-day diabetic rats (Shibata, 1987). In 2-day diabetic rats, tissue protein or cytochrome oxidase activity in BAT were decreased only at 70% of the level of those in the control rats. It is likely that cold-induced thermogenic capacity may be still preserved in 2-day diabetic rats. In our long-term diabetic rats, however, the levels of these parameters markedly decreased to below about the 40% level of those in the control rats. This may have contributed to the impairment of acute cold-induced thermogenesis.

The work of others has demonstrated a marked increase in the sensitivity to insulin induced by cold-exposure (Vallerand *et al.*, 1986, 1987; Bartness *et al.*, 1986a, 1986b). Cold-exposure potentiates the effect of insulin on *in vivo* glucose uptake in BAT at a normal insulin level, and glucose would principally be utilized in de novo lipogenesis as fuel for BAT thermogenesis. In insulin-deficient diabetic rats, however, the uptake of glucose in BAT would be impaired even in cold-exposure.

In conclusion, the present study demonstrates that insulin is not essential in potentiating NE turnover in BAT after acute cold-exposure, but is required for cold-induced thermogenesis.

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