Trace metal, acute phase and metabolic response to endotoxin in metallothionein-null mice

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Accumulation of hepatic zinc via metallothionein (MT) induction during infection/inflammation is postulated to benefit a range of metabolic processes. The metabolic consequences of two doses of endotoxin (LPS) (1 and 5 mg/kg, intraperitoneally) were examined in normal (MT+/+) and MT-null (MT−/−) mice (all results means ± S.E.M., $n = 6$). At 16 h after 1 mg/kg LPS, hypozincaemia was pronounced in the MT+/+ mice (4.4 ± 0.2 μM), concomitant with a 36% increase in hepatic Zn and a >10-fold increase in hepatic MT. Plasma Zn (16.6 ± 0.7 μM) and total hepatic Zn were unchanged in MT−/− mice, confirming the importance of MT in altering plasma and hepatic Zn during inflammation. Plasma iron was lower in LPS-treated MT−/− mice, whereas plasma copper increased to a similar extent in both groups of mice. Plasma fibrinogen more than doubled, and was similar in both groups of mice, which questions the importance of MT in acute-phase protein synthesis. Blood and liver glucose concentrations were not significantly different between groups before or after LPS, whereas blood and liver lactate concentrations were significantly lower (31% and 24% respectively) in MT−/− mice after LPS. At 16 h after 5 mg/kg LPS, plasma Zn was decreased even further in MT+/+ mice (2.6 ± 0.3 μM), but remained unchanged in MT−/− mice at concentrations significantly above those in 16 h-fasted MT−/− mice (15.8 ± 0.5 versus 11.3 ± 0.3 μM). Total liver Zn was 17% lower than fasting values in MT−/− mice, in contrast with 32% higher in MT+/+ mice. Synthesis of MT (in MT+/+ mice) and fibrinogen in all mice was not further enhanced by the higher LPS dose. Blood glucose was significantly decreased by 18% in MT+/+ mice and by 38% in MT−/− mice after 5 mg/kg LPS. There was a marked 44% decrease in liver glucose in MT−/− mice; that in MT+/+ mice was unchanged from fasting levels, implying a deficit in hepatic gluconeogenesis in LPS-treated MT−/− mice. In the absence of any indication of major hepatotoxicity, the results of this study indicate that energy production, and not acute-phase protein synthesis, may be most influenced by Zn supply during endotoxaemia, suggesting that MT has a role in maintaining hepatic and blood glucose in this metabolic setting.

INTRODUCTION

The cysteine-rich metal-binding protein metallothionein (MT) has been ascribed broad functions in the regulation of Zn and Cu homoeostasis, participation in the acute-phase response, detoxification of heavy metals and scavenging of free radicals [1–4]. The generation of mice lacking expression of MT-1 and MT-2 genes [5,6] has enabled the more rigorous investigation of MT. Current evidence indicates that MT−/− mice reproduce and grow normally, but are sensitive to the toxic effects of Cd [5,6]. We have previously reported that in MT−/− mice hepatic Zn does not rise after intraperitoneal administration of endotoxin (LPS) [7] or $ZnSO_4$ [8], two potent stimulators of hepatic MT synthesis and liver Zn uptake in normal mice. This raised a question regarding the metabolic impact of an inability to accumulate hepatic Zn-MT during acute inflammation, where there is an obligatory requirement for a net increase in protein synthesis as well as major changes in fuel usage. It was apparent from our initial studies that MT−/− mice can withstand moderate doses of LPS (1 mg/kg) with adverse effects no greater than those typical of normal mice (anorexia, piloerection). As induction of hepatic MT occurs at the onset of inflammation [1–3], it has been suggested that the resulting increased supply of exchangeable Zn may facilitate the many enzymic processes necessary for mounting the acute-phase response. Given that glucocorticoids, glucagon, catecholamines and cytokines (interleukin-6 in particular) not only induce MT [9–11] and acute-phase protein synthesis [12,13], but also strongly influence hepatic metabolism [11,14,15], the hepatic accumulation of Zn during infection/inflammation would be expected to be important in maintaining energy metabolism in the liver.

A close relationship between hepatic MT and plasma fibrinogen and caeruloplasmin has been described [16] in support of a link between MT induction and the acute-phase response. In the metabolic setting, Zn is important in many enzymic reactions, with clear demonstrations that Zn sequestered by MT can be donated to Zn-dependent enzymes *in vitro* [17]. An increased supply of hepatic Zn may thus be essential in facilitating metabolic processes in the gluconeogenic/glycolytic pathway when large energy demands are placed on the host. A failure to accumulate Zn would therefore be expected to have deleterious effects on hepatic metabolism. In this study, we examined the effect of LPS-induced inflammation on plasma levels of the acute-phase protein, fibrinogen, in MT+/+ and MT−/− mice. In addition, we have investigated a link between energy metabolism, as reflected by blood and liver substrate concentrations, and altered Zn homoeostasis during both moderate and severe LPS-induced inflammation.

MATERIALS AND METHODS

MT-I and MT-II null mice were produced by A. Michalska and K. H. A. Choo of the Murdoch Institute, Royal Childrens Hospital, Victoria [5]. The mice were in a mixed genetic background of OLA129 and C57BL6 strains. Control mice (MT+/+) were C57BL6, supplied by the Animal Resource Centre, Canning Vale, Western Australia. All mice were housed on sawdust in stainless-steel or plastic cages in an animal house at

Abbreviations used: MT, metallothionein; LPS, endotoxin (lipopolysaccharide); LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PEPCK, phosphoenolpyruvate carboxykinase.

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22 °C with a 14 h-light/10 h-dark cycle and were fed on a commercial pelleted diet. Experimental details have recently been described [7]. In brief, mice (23–29 g) were injected (intraperitoneally) with lipopolysaccharide (LPS; from *Escherichia coli* 0111.B4; Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dosage of 1 or 5 mg/kg body wt. in 0.15 ml of 0.85 M NaCl. Food was withheld, but free access to water was allowed. At 16 h after intraperitoneal LPS injection, the mice were anaesthetized with halothane and 1 ml of blood was withdrawn by cardiac puncture. The mice were then killed by cervical luxation, and a portion of the liver was immediately homogenized in 4 vol. of ice-cold 5 % $HClO_4$. The remainder of the liver was retained for MT assay [7,18,19]. The kidneys and spleen were removed and weighed. Whole blood was deproteinized in 4 vol. of ice-cold 5 % $HClO_4$ and, after centrifugation, metabolites were measured in neutralized supernatants (blood and liver) by using enzyme-based assays as previously described [20]. Zn and Cu were analysed by atomic-absorption spectroscopy on plasma samples and dried liver homogenates subjected to HNO_3 digestion (Zn only). MT was measured in the wet liver homogenates [7]. Plasma fibrinogen was measured with a Cobas Fibro analyser using Fibriquik thrombin reagent (Organon Technika) and plasma Fe with a Cobas Bio analyser using Iron FZ kits (F. Hoffman–La Roche and Co. Diagnostica, Basle, Switzerland). All experimentation was approved by the IMVS Animal Ethics Committee. Results are reported as means ± S.E.M. Significance was determined by Student's *t* test for independent samples.

RESULTS

Changes in body and organ weights (Table 1)

Weight loss was not significantly different between any of the groups, except for the MT−/− mice given 5 mg/kg LPS, which lost significantly less weight than did all other groups. It was noted that the stomachs of mice in this group were heavier and the contents more fluid than in other groups. As food had been withheld, it was assumed that the body-weight difference reflected increased water consumption in these mice. LPS treatment was associated with a significant increase in liver weight in all groups except the MT−/− mice treated with 5 mg/kg LPS. Kidney weights were the same in all groups. There was a small but significant increase in spleen weights after 1 mg/kg LPS treatment in both MT+/+ and MT−/− mice, but only the MT+/+ mice showed this effect at 5 mg/kg LPS.

Changes in plasma zinc, copper, iron and fibrinogen (Table 2)

Plasma Zn did not decrease in LPS-treated MT−/− mice compared with the normal mean concentration in fed MT−/− mice of 14.5 ± 0.5 μM ($n = 26$), whereas gross hypozincaemia occurred in MT+/+ mice [4.4 μM and 2.6 μM at 1 mg/kg and 5 mg/kg doses of LPS respectively; fed plasma Zn for MT+/+ mice 13.24 ± 0.30 μM ($n = 24$)]. Fasting alone resulted in a significantly greater decrease in plasma Zn concentrations in MT−/− mice, to 11.3 μM, whereas plasma Zn concentrations were maintained in MT+/+ mice. LPS (1 mg/kg) elicited an increase in plasma Cu concentrations in both MT+/+ and MT−/− mice, which was not observed at 5 mg/kg LPS. Plasma Fe concentrations decreased respectively by 37 % and 60 % in response to 1 mg/kg and 5 mg/kg LPS in MT−/− mice, in contrast with the plasma Zn response. The increase in plasma fibrinogen was used to assess the acute-phase response. At 16 h after LPS administration (1 and 5 mg/kg dose) there was a similar increase in plasma fibrinogen in both MT+/+ and MT−/− groups.

To assess the extent of toxicity of LPS treatment, the activities of lactate dehydrogenase (LDH), alanine aminotransferase

Table 1 Effect of LPS on body and organ weights

All mice were fasted for 16 h. Results are shown as means ± S.E.M. ($n = 6$): *significantly different from control (16 h fast, no treatment), at $P < 0.05$; †MT−/− significantly different from MT+/+ within a treatment, at $P < 0.05$.

Treatment	Mice	Initial body wt. (g)	Wt. loss (%)	Organ wt. (% of initial body wt.)		
				Liver	Kidney	Spleen
None (16 h fast)	MT+/+	26.3 ± 0.4	−8.19 ± 0.30	3.37 ± 0.13	1.27 ± 0.03	0.26 ± 0.02
	MT−/−	27.6 ± 0.6	−9.23 ± 0.65	3.66 ± 0.08	1.30 ± 0.05	0.22 ± 0.01
LPS 1 mg/kg	MT+/+	25.4 ± 0.6	−8.95 ± 0.48	4.08 ± 0.05*	1.30 ± 0.06	0.33 ± 0.01*
	MT−/−	25.1 ± 0.9	−9.81 ± 0.83	4.33 ± 0.11*	1.30 ± 0.04	0.36 ± 0.03*
LPS 5 mg/kg	MT+/+	25.4 ± 0.4	−9.97 ± 0.79	4.42 ± 0.23*	1.40 ± 0.09	0.35 ± 0.01*
	MT−/−	25.1 ± 0.4	−5.80 ± 0.92*	3.58 ± 0.12†	1.27 ± 0.07	0.28 ± 0.02†

Table 2 Effect of LPS on plasma metal and fibrinogen concentrations

All mice were fasted for 16 h. Results are shown as means ± S.E.M. ($n = 6$): *significantly different from control (16 h fast, no treatment), at $P < 0.05$; †MT−/− significantly different from MT+/+ within a treatment, at $P < 0.05$.

Treatment	Mice	Zn (μM)	Fe (μM)	Cu (μM)	Fibrinogen (g/l)
None (16 h fast)	MT+/+	15.0 ± 0.1	11.1 ± 0.7	9.2 ± 0.9	1.76 ± 0.05
	MT−/−	11.3 ± 0.3†	17.6 ± 1.5†	9.2 ± 0.3	1.94 ± 0.05†
LPS 1 mg/kg	MT+/+	4.4 ± 0.2*	14.5 ± 0.3*	14.4 ± 0.7*	4.49 ± 0.26*
	MT−/−	16.6 ± 0.7	11.1 ± 1.6*†	17.1 ± 2.4*	4.14 ± 0.16*
LPS 5 mg/kg	MT+/+	2.6 ± 0.3*	8.9 ± 0.3*	8.3 ± 0.5	4.53 ± 0.31*
	MT−/−	15.8 ± 0.5	7.0 ± 0.6*†	10.4 ± 0.4	3.73 ± 0.19*

Table 3 Effect of LPS on hepatic zinc and MT concentrations

All mice were fasted for 16 h. Results are shown as means ± S.E.M. ($n = 6$). In fed mice, total hepatic Zn (in nmol); MT+/+ 558 ± 20 ($n = 16$), MT−/− 559 ± 39 ($n = 18$). *Significantly different from control (16 h fast, no treatment), at $P < 0.05$; †MT−/− significantly different from MT+/+ within a treatment, at $P < 0.05$.

Treatment	Mice	Zinc (nmol/total liver)	MT (nmol of Cd bound/total liver)
None (16 h fast)	MT+/+	448 ± 30	21 ± 1
	MT−/−	428 ± 20	< 2†
LPS 1 mg/kg	MT+/+	629 ± 19*	90 ± 8*
	MT−/−	471 ± 22†	< 2†
LPS 5 mg/kg	MT+/+	592 ± 46*	83 ± 5*
	MT−/−	353 ± 16*†	< 2†

(ALT) and aspartate aminotransferase (AST), assayed by standard UV methods [20a], were measured in the plasma of the mice treated with 5 mg/kg LPS. A 2-fold increase in all enzymes was observed, compared with fasted controls, with no difference between MT+/+ and MT−/− mice: LPS-treated, MT+/+ LDH, 300 ± 23; ALT, 23 ± 5; AST, 57 ± 7; LPS-treated, MT−/− LDH, 347 ± 23; ALT, 27 ± 4; AST, 69 ± 6; (normal levels: LDH, 136 ± 7; ALT, 15 ± 3; AST, 27 ± 1; all values U/L, means ± S.E.M., $n = 6$).

Hepatic zinc and MT (Table 3)

Total liver Zn in LPS-treated MT+/+ mice was 33–40 % higher than in the fasted controls. Zn did not accumulate in the livers of MT−/− mice at 1 mg/kg LPS, and decreased significantly (17 %) after treatment with 5 mg/kg LPS. In MT+/+ mice, fasting caused a 3–4-fold increase in liver MT (basal values were 5 nmol of Cd bound/liver), whereas LPS treatment caused a 15–18-fold increase. The effect of 1 mg/kg and 5 mg/kg LPS was not significantly different in this regard. Kidney MT levels paralleled the hepatic changes, although the concentrations were much lower (results not shown). MT−/− mice had Cd binding at the level of non-specific binding in the Cd–haem assay. The inability of these mice to synthesize MT has been well substantiated [5,7].

Changes in blood and liver substrate concentrations (Table 4)

Blood lactate and glucose concentrations were not significantly different between groups of fasted MT+/+ and MT−/− mice. Although blood lactate concentrations were significantly increased in both groups of mice after 1 mg/kg LPS, the difference in the increment between the MT+/+ (97 % increase) and MT−/− mice (37 % increase) was also significant. LPS at 5 mg/kg elicited the same response in lactate concentrations in MT+/+ and MT−/− mice (32–33 % increase). At 5 mg/kg LPS, blood glucose concentrations were significantly decreased by 18 % and 38 % in MT+/+ and MT−/− mice respectively. Liver lactate concentrations demonstrated similar changes to those in blood, with a lower hepatic lactate accumulation in MT−/− mice after fasting or LPS treatment. Glucose concentrations in the livers of MT+/+ and MT−/− mice were not significantly different between fasting and 1 mg/kg LPS treatment, and the increase in liver lactate concentration after LPS treatment was not significantly different between 1 mg/kg and 5 mg/kg LPS. Liver glucose concentrations, on the other hand, decreased by 44 % in MT−/− mice, but were unaffected in MT+/+ mice at 5 mg/kg LPS. To assess the level of fat catabolism, liver hydroxybutyrate concentrations were measured in mice which were fasted or had received 5 mg/kg LPS. Sample limitations precluded the measurement of acetoacetate, but our previous studies with fasted mice have confirmed that hydroxybutyrate is the predominant ketone body. Liver hydroxybutyrate concentrations were significantly higher in MT−/− mice than in MT+/+ mice in response to fasting (3.48 ± 0.19 versus 1.05 ± 0.20 μmol/g). After LPS (5 mg/kg) there was no further increase in hydroxybutyrate in MT−/− mice (4.24 ± 0.54 μmol/g), but a significant increase in the MT+/+ mice (2.70 ± 0.37 μmol/g), although still lower than in the MT−/− mice. As the hydroxybutyrate concentrations indicated a more rapid shift to the fasting state in MT−/− mice, glycogen levels were measured in fed mice. MT+/+ mice had significantly higher glycogen concentrations than MT−/− mice [168 ± 11 versus 132 ± 7 ($n = 6$) μmol glucose equivalents/g; $P < 0.05$].

DISCUSSION

It has long been known that during major disease (infection being the archetype) there are marked changes in trace-metal, endocrine-hormone and whole body metabolism, processes linked to cytokine release [14,15]. The immediate metabolic response of the liver to infection/inflammation is a shift towards catabolism, the liver's primary function being that of an energy tranformer. Initially, glycogen is metabolized to glucose to sustain glycaemia in response to decreased food intake, and fat oxidation is increased to provide energy and alternative fuels (ketone bodies). Following glycogen depletion, glycaemia can only be maintained by gluconeogenesis from lactate and amino acids.

Table 4 Blood and liver metabolite concentrations

LPS-treated mice were also fasted for 16 h. Results are shown as means ± S.E.M. ($n = 6$): *significantly different from fasted, at $P < 0.05$; †MT−/− significantly different from MT+/+ within a treatment, at $P < 0.05$.

		Blood (mM)		Liver (μmol/g wet wt.)	
Treatment	Mice	Glucose	Lactate	Glucose	Lactate
None (16 h fast)	MT+/+	5.00 ± 0.24	0.98 ± 0.09	12.16 ± 1.68	3.51 ± 0.18
	MT−/−	5.44 ± 0.16	0.97 ± 0.05	11.87 ± 2.49	2.25 ± 0.35†
LPS 1 mg/kg	MT+/+	5.51 ± 0.04	1.93 ± 0.16*	12.74 ± 0.75	8.52 ± 0.71*
	MT−/−	5.76 ± 0.27	1.33 ± 0.09*†	11.39 ± 1.49	6.50 ± 0.78*†
LPS 5 mg/kg	MT+/+	4.08 ± 0.03*	1.30 ± 0.08*	12.07 ± 1.95	6.60 ± 0.69*
	MT−/−	3.38 ± 0.24*	1.28 ± 0.09*	6.68 ± 0.61*†	5.34 ± 0.74*

Priorities for amino acid metabolism must be balanced between albumin synthesis, acute-phase protein synthesis and energy metabolism. Although a link between hepatic Zn accumulation via MT induction and acute-phase protein synthesis has been suggested, the evidence presented in this study indicates that metabolic events central to energy production, and not acute-phase protein synthesis, may be influenced by Zn supply during endotoxaemia.

A close relationship between hepatic MT and plasma fibrinogen and caeruloplasmin has been reported [16]. In the present study, MT−/− mice equalled MT+/+ mice in fibrinogen production in response to 1 and 5 mg/kg LPS, despite lacking MT and hence having much lower hepatic Zn levels. Although other acute-phase proteins show dramatic responses to inflammatory stimuli (e.g. serum amyloid A rises from low levels to 1 g/l [35]), the alteration in fibrinogen is one of the most prominent in terms of mass (changes of 2–4 g/l [16]), and would be expected to be affected if protein synthesis was limited by a failure to accumulate hepatic Zn. Plasma Cu levels increased in both groups of mice in response to 1 mg/kg LPS, presumably as a result of caeruloplasmin export from the liver. This increase in plasma Cu was not observed in MT−/− or MT+/+ mice after 5 mg/kg LPS, which suggests that caeruloplasmin and fibrinogen respond differently to the higher dose of LPS. It is concluded that the previous correlation observed between MT and fibrinogen [16] reflects similar dose–responses to a common effector, probably interleukin-6 [9], rather than a direct link between Zn supply and induction of this acute-phase protein. The indication that MT−/− mice respond normally to the stimulus of acute-phase protein production, as indicated by the increase in fibrinogen, points to the need for a closer examination of the proposed interrelationships between Zn and acute-phase protein synthesis, particularly as metal-responsive elements have now been described for a number of acute-phase protein genes [34].

In confirmation of our earlier study [7], MT−/− mice tolerated moderate doses of LPS (1 mg/kg) with mild adverse effects typical of MT+/+ mice (anorexia, piloerection). This was sufficient to elicit a maximal MT response in MT+/+ mice, as demonstrated by no additional increase in hepatic MT or Zn with a 5-fold higher dose. Examination of other indices, including weight loss and alterations in organ weights, did not reveal any major differences between the two groups of mice at 1 mg/kg LPS. There were, however, significant liver weight, spleen weight and metabolic differences between MT+/+ and MT−/− mice at 5 mg/kg LPS that were not apparent at the lower LPS dose, and which coincided with a decrease in hepatic Zn concentrations and a more marked decrease in total liver Zn in the MT−/− mice.

In the metabolic setting, Zn is important in many enzymic reactions, with evidence that Zn sequestered by MT can be donated to Zn-dependent enzymes *in vitro* [17]. For example, fructose 1,6-bisphosphatase is a key gluconeogenic enzyme, which responds to changes in Zn and has been shown to interact with other Zn-binding proteins [21–23]. Alteration in the activity of this and other enzymes in the gluconeogenic/glycolytic pathway could regulate energy metabolism during inflammation. Of prime importance in this study was a 44% decrease in liver glucose concentration after 5 mg/kg LPS treatment in MT−/− mice, which, combined with the low blood glucose concentration in these mice (3.4 mM; cf. 5.4 mM with fasting alone), implies a deficit in hepatic gluconeogenesis.

Decreased hepatic gluconeogenesis has frequently been observed after LPS treatment, an observation linked with altered transcriptional control of phosphoenolpyruvate carboxykinase (PEPCK), a key rate-limiting enzyme in the gluconeogenic pathway [24]. Most recently, experiments with isolated rat hepatocytes have indicated that interleukin-6 inhibits the hormone-induced (glucagon and insulin) expression of this enzyme, and furthermore decreases the stability of the mRNA for PEPCK [25]. In mouse liver, interleukin-1 causes a decrease in corticosterone-induced PEPCK activity, a finding linked to down-regulation of intracellular steroid receptors [26]. A confounding factor in this setting is that insulin, as well as glucagon, is often raised during endotoxaemia, with opposing effects on PEPCK [24,25]. However, the high level of fat catabolism, as indicated by the elevated hepatic hydroxybutyrate concentrations in the MT−/− mice, suggests an over-riding effect of glucagon.

Comparison of MT+/+ and MT−/− mice after fasting indicates that the MT−/− mice move more rapidly into a fasting state. Given the finding of lower glycogen levels in the MT−/− mice before fasting, it would be expected that MT−/− mice would become glycogen-depleted earlier after fasting or LPS treatment, with a consequent earlier decrease in blood glucose and increase in blood ketone-body concentrations. This is consistent with the metabolic pattern seen at 16 h after LPS treatment, which is characterized by accelerated fat catabolism (increased ketone bodies) and lower blood and liver glucose concentrations. Investigations into the temporal relationship between glycogen depletion, blood glucose and ketone-body concentrations, insulin and glucagon levels and glucose turnover are warranted to elucidate this interesting metabolic difference in MT−/− mice**.** There is evidence that MT has a role in preserving body Zn reserves during starvation [27,28], in response to a decreased insulin:glucagon ratio [29,30]. However, we observed that MT+/+ and MT−/− lost equal amounts of hepatic zinc on fasting, despite a 3–4-fold increase in MT in the MT+/+ mice. During endotoxaemia, MT−/− mice clearly cannot accumulate Zn in the liver, and actually lose hepatic Zn at a high dose of LPS, coinciding with a marked decrease in hepatic and blood glucose concentrations. Closer examination of a possible causal relationship between a failure to accumulate hepatic Zn and metabolic change is warranted.

Although MT synthesis is responsible for the inflammation-induced increase in hepatic Zn, MT has also to be ascribed a role in protecting the cellular environment from reactive oxygen species. There is mounting evidence that MT protects the liver against a range of compounds which induce oxidative stress, including cytokines and LPS [31,32]. The metabolic effects in MT−/− mice may thus relate to a lack of antioxidant function in the absence of MT, resulting in a more toxic direct effect of LPS [33] or indirect effect via cytokines [32,33]. Evidence from the present study does not support this explanation, as the increase in plasma LDH, ALT and AST was minor (2-fold increase) and similar in MT+/+ and MT−/− mice after 5 mg/kg LPS. It is possible that damage to membranes is the end result of altered cellular metabolism and that the findings in this paper reflect early events which are linked to the absence of MT and the resultant failure to accumulated hepatic Zn after exposure to LPS.

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