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The Beneficial Effect of Trolox on Sepsis-Induced Hepatic Drug Metabolizing Dysfunction

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Trolox is a hydrophilic analogue of vitamin E. The aim of this study was to investigate its effects on hepatic injury, especially alteration in cytochrome P450 (CYP)-dependent drug metabolism during polymicrobial sepsis. Rats were subjected to polymicrobial sepsis by cecal ligation and puncture (CLP). The rats were treated intravenously with Trolox (2.5 mg/kg) or vehicle, immediately after CLP. Serum aminotransferases and lipid peroxidation levels were markedly increased 24 h after CLP. This increase was attenuated by Trolox. Total CYP content and NADPH-P450 reductase activity decreased significantly 24 h after CLP. This decrease in CYP content was attenuated by Trolox. At 24 h after CLP, there was a significant decrease in the activity of these CYP isozymes: CYP1A1, 1A2, 2B1, and 2E1. However, Trolox differentially inhibited the decrease in CYP isozyme activity. Trolox had little effect on the decrease in CYP1A1 activity but Trolox significantly attenuated decreases in CYP1A2 and 2E1 activities . In fact, Trolox restored CYP2B1 activity to the level of activity found in control rats. Our findings suggest that Trolox reduces hepatocellular damage as indicated by abnormalities in hepatic drug-metabolizing function during sepsis. Our data also indicates that this protection is, in part, caused by decreased lipid peroxidation.

Key words: Trolox, Polymicrobial sepsis, Lipid peroxidation, Cytochrome P450 isozyme activities

INTRODUCTION

Sepsis and systemic inflammatory response syndrome (SIRS) continue to be the most common causes of morbidity and mortality in intensive care units, despite various therapeutic advances in the management of sepsis (Baue, 1994; Barriere and Lowry, 1995). Sepsis therefore continues to have significant clinical implications and remains an area that attracts intense research interest. It is well known that the liver plays a major role in the clearance of systemic toxemia and is postulated as a regulatory organ in the host-defense system (Koichi *et al.*, 2001). Although hepatocellular dysfunction occurs during sepsis (Dhainaut *et al.*, 2001), the specific sequence of events that leads to the damage is still not clear.

Increased production of reactive oxygen species (ROS), whether from trauma, ischemia, or infection, leads to oxidative stress by many mechanisms. Furthermore, there is evidence that implicates oxidative stress in the pathogenesis of septic shock and multiple organ dysfunction syndromes (MODS) (Horton, 2003; Goode *et al.*, 1995). ROS are capable of interacting with a wide range of biomolecules leading to lipid peroxidation of cell membranes, increased membrane permeability, and ultimately cell death. It has been reported that exogenously added free radical scavengers slow down deterioration of cell function caused by endotoxic shock (Adel *et al.*, 2001; Varda *et al.*, 2001).

The alteration of drug metabolism under pathophysiological conditions is clinically important. Hepatic cytochrome P450 (CYP) isozymes play a key role in the metabolism of clinically important drugs (Porter and Coon, 1991). In patients with chronic liver disease, the elimination of drugs metabolized by the liver is often impaired (Huet and Villenueve, 1983). In addition, many reports have shown that several hepatic CYP isozymes were down-regulated during systemic infection (Kokwaro *et al.*, 1993; Topfer *et*

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al., 1995). Indeed, our previous studies demonstrated that abnormalities occur in microsomal drug-metabolizing function during hepatic ischemia and reperfusion *in vivo*. Hepatic secretory function is also affected by ischemia and reperfusion. These liver malfunctions were found to be associated with lipid peroxidation However, direct association has not been established between microsomal lipid peroxidation *in vivo* after sepsis and changes in the activity of CYP isozymes.

Trolox is a water-soluble analogue of vitamin E. Trolox has been reported to be an excellent antioxidant *in vitro* (Doba *et al.*, 1985; Barkley *et al.*, 1985). In SDS micelles, Trolox was shown to scavenge peroxyl radicals eight times better than α-tocopherol (Castle and Perkins, 1986). However, the precise mechanism of the *in vivo* antioxidant effect of Trolox remains unclear.

Therefore, the purpose of this study was to investigate the effect of Trolox on hepatic injury during polymicrobial sepsis, particularly on the deterioration of CYP-dependent microsomal drug metabolizing function.

MATERIALS AND METHODS

Chemicals

Trolox was supplied by the Aldrich Chemical Co. (Gillingham, UK). Ethoxyresorufin, methoxyresorufin, pentoxyresorufin, aniline, NADPH, HEPES, and thiobarbituric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade and were locally and commercially available.

Animals

Male Sprague-Dawley rats weighing 260-300 g were obtained from Jeil animal breeding company of Korea and were acclimatized to laboratory conditions at Sungkyunkwan University for at least one week. Rats were kept in a temperature and humidity controlled room (25 ± 1°C and 55 ± 5%, respectively) with 12 h of light-dark cycle.

Cecal ligation and puncture (CLP)

Polymicrobial sepsis in rats was induced by CLP according to the method of Chaudry *et al.* (1979). Rats were fasted overnight, but allowed to drink tap water *ad libitum.* After anesthetized by intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (20 mg/kg), a 15-mm ventral midline incision was performed. The cecum was then exposed, ligated just distally to ileocecal valve to avoid intestinal obstruction, punctured twice with an 18-gauge needle, and returned to the abdominal cavity. The abdominal incision was then closed in 2 layers, and the animals subcutaneously received normal saline solution, 3 mL per 100 g of body weight. Sham-operated animals (control) underwent the same surgical procedure without CLP. All animals were studied at 24 h after the onset of sepsis.

Administration of Trolox

Trolox, dissolved in phosphate buffered saline (PBS, pH 7.4), was administered by intravenous injection at a dose of 2.5 mg/kg of body weight, immediately after the CLP procedure. In the vehicle-treated rats, PBS was injected in the same volume and manner as Trolox. Four experimental groups were studied: (a) vehicle-treated control (control), (b) Trolox-treated control (Trolox), (c) vehicle-treated CLP, and (d) Trolox-treated CLP.

Isolation of hepatic microsomal fraction

Liver samples were removed and placed in ice-cold 0.9% NaCl solution. They were then weighed, minced, and homogenized with a teflon pestle homogenizer in 4 volumes of homogenizing buffer containing 1.15% KCl and 50 mM Tris HCl (pH 7.4). The whole homogenate was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was collected and centrifuged at 105,000 g for 60 min at 4°C. Microsomal precipitates of 1 g of liver microsome were resuspended in 10 volumes of storage buffer containing 15% KCl, 10 mM HEPES, and 1 mM EDTA (pH 7.6)and stored at -70°C until assayed. The content of microsomal protein was determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard.

Analytical procedures

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by standard spectrophotometric procedure using Sigma Kit 52-UV and 51-UV (Sigma Chemical Co., St. Louis, MO, USA), respectively. Microsomal lipid peroxidation in the liver was estimated from thiobarbituric acid reactant levels using the assay method of Buege and Aust (1978). Total CYP content was calculated by using a molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ for the absorbance difference between 450 and 490 nm in a differential spectrophotometer (Omura and Sato, 1964). NADPH-P450 reductase activity was determined by its NADPH-cytochrome *c* reductase activity (Vermillion and Coon, 1978). Aniline *p*-hydroxylase activity (CYP2E1) was determined by measuring the formation of *p*-aminophenol (Schenkman *et al.*, 1967). Activities of CYP1A1, 1A2, and 2B1 in liver microsomal fraction were measured by the method of Burke *et al.* (1985). CYP1A1 was measured as activity of 7-ethoxyresorufin β-deethylase (EROD), methoxyresorufin β-demethylase (MROD) represented 1A2 activity, and 2B1 activity was determined using pentoxyresorufin β-dealkylase (PROD) activity The reaction mixture contained 100 mM Tris-HCl buffer at pH 7.5, 25 mM $MgCl_2$, 5 μM substrates (ethoxy-, methoxy-, or pentoxyresorufin) and microsome.

The reaction was initiated by the addition of 1 mM NADPH and incubated at 37°C for 10 min. After incubation, reactions were terminated by adding methanol and the mixtures were centrifuged at 2,000 g for 10 min. Fluorescence of resorufin in the supernatant was measured at excitation and emission wavelengths of 550 and 580 nm, respectively.

Statistics

All data are expressed as means ± SEM. Overall significance was tested by one-way analysis of variance followed by Dunnett's *t*-test, and the significance level was set at $p<0.05$.

RESULTS

Mortality

Mortality was 17% (2 dead/12 total) after CLP. Treatment with Trolox decreased mortality from 17% to 0% (0 dead/12 total) after CLP. There was no mortality in vehicle-treated control (0 dead/12 total) or Trolox-treated control rats (0 dead/12 total).

Serum ALT and AST

The serum levels of ALT in the vehicle-treated and Trolox-treated control rats were 39.0 ± 1.7 and 36.1 ± 3.0 U/L, respectively. At 24 h after CLP, the serum ALT activity increased to 58.4 ± 5.3 U/L. This increase was not attenuated by Trolox. However, at 24 h after CLP, the serum AST activity increased from 141.7 ± 8.5 U/L to 236.0 ± 13.4 U/L. This increase was significantly attenuated by Trolox (Table I).

Lipid peroxidation

The results of malondialdehyde (MDA) determination are presented in Table I. In the vehicle-treated and Trolox-treated control rats, the levels of MDA in liver microsomes were 1.12 ± 0.05 and 1.15 ± 0.06 nmol/mg protein/min, respectively. On the other hand, at 24 h after CLP, the MDA level markedly increased to 1.68 ± 0.07 nmol/mg protein/min. This elevation was significantly attenuated by Trolox.

Table I. Effect of Trolox on serum aminotransferases and lipid peroxidation during CLP

Group	ALT (U/L)	AST (U/L)	Malondialdehyde (nmol/mg protein/min)
Control	39.0 ± 1.7	141.7 ± 8.5	1.12 ± 0.05
Trolox	36.1 ± 3.0	135.7 ± 6.2	1.15 ± 0.06
CLP	58.4 ± 5.3**	236.0 ± 13.4**	1.68 ± 0.07**
CLP+Trolox	48.8 ± 2.9	184.3 ± 8.2**++	1.24 ± 0.05++

** = significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from CLP. Values are means ± SEM for 10-12 rats per group.

Total CYP content and NADPH-P450 reductase activity

As shown in Fig. 1, the total hepatic microsomal CYP content in the vehicle-treated control rats was 0.46±0.04 nmol/mg protein. No changes were observed in P450 content in Trolox-treated control rats compared with vehicle-treated control rats. However, CLP markedly reduced it to 0.23 ± 0.02 nmol/mg protein. The decrease in CYP content was attenuated by Trolox. Similar to CYP content, the hepatic microsomal NADPH-P450 reductase activity significantly decreased after CLP. This decrease was not attenuated by Trolox (Fig. 2).

CYP isozyme activity

As shown in Fig. 3 the activity of CYP1A1 in the vehicle-

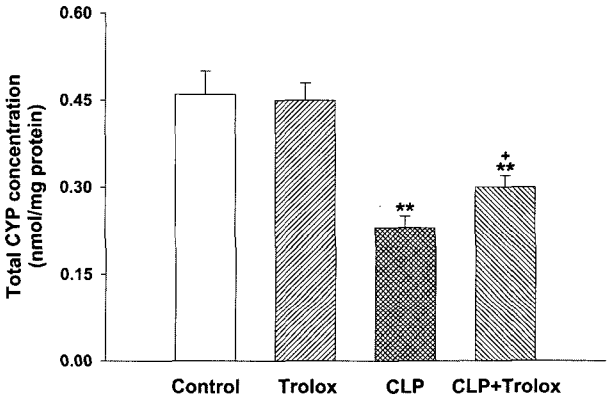

Fig. 1. Effect of Trolox on hepatic microsomal cytochrome P450 concentration during CLP. ** = Significantly different ($p<0.01$) from controls. + = Significantly different ($p<0.05$) from CLP. Values are means ± SEM for 10-12 rats per group.

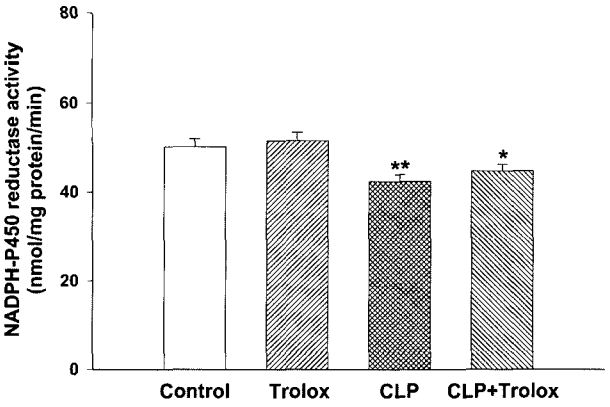

Fig. 2. Effect of Trolox on hepatic microsomal NADPH-cytochrome P450 reductase activity during CLP. *,** = Significantly different ($p<0.05$, $p<0.01$) from controls. Values are means ± SEM for 10-12 rats per group.

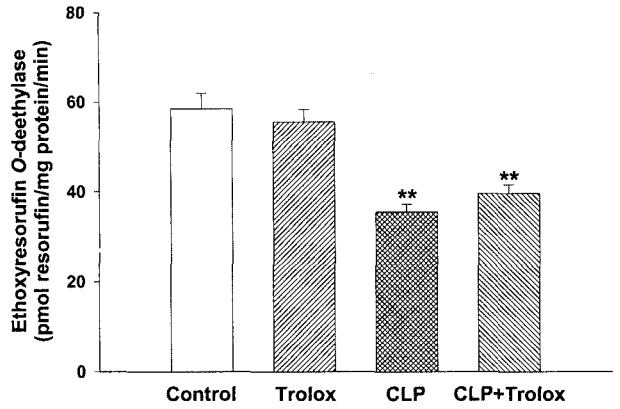

Fig. 3. Effect of Trolox on hepatic microsomal CYP1A1 activity during CLP. ** = Significantly different ($p<0.01$) from controls. Values are means ± SEM for 10-12 rats per group.

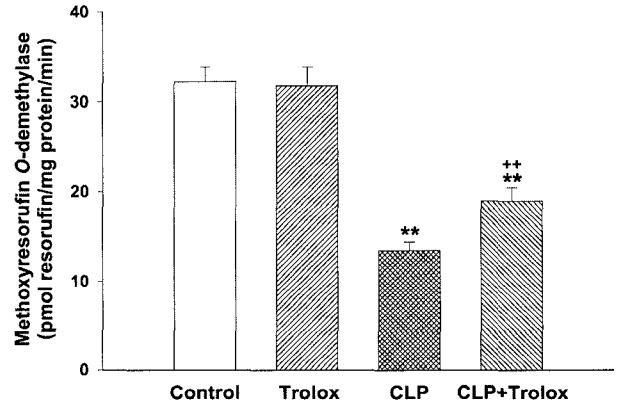

Fig. 4. Effect of Trolox on hepatic microsomal CYP1A2 activity during CLP. ** = Significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from CLP. Values are means ± SEM for 10-12 rats per group.

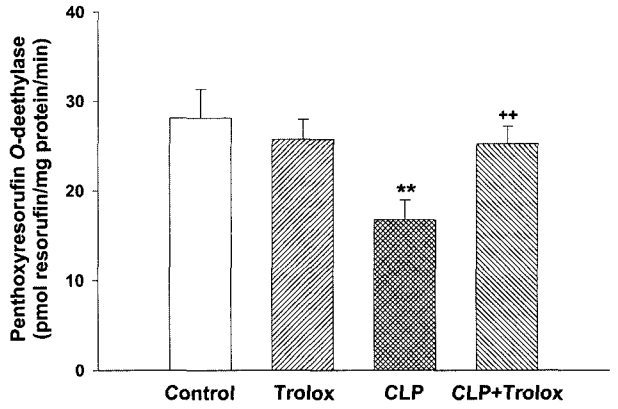

Fig. 5. Effect of Trolox on hepatic microsomal CYP2B1 activity during CLP. ** = Significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from CLP. Values are means ± SEM for 10-12 rats per group.

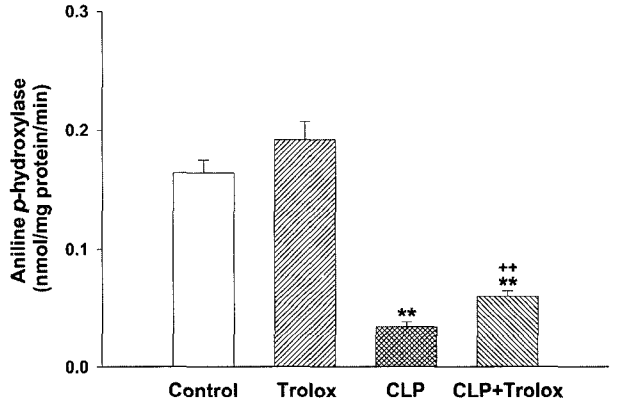

Fig. 6. Effect of Trolox on hepatic microsomal CYP2E1 activity during CLP. ** = Significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from CLP. Values are means ± SEM for 10-12 rats per group.

treated and Trolox-treated control rats was 58.5 ± 3.5 and 55.6 ± 2.8 pmol/mg protein/min, respectively. At 24 h after CLP, CYP1A1 activity decreased to 35.6 ± 1.8 pmol/mg protein/min. Trolox had little effect on the decrease in CYP1A1 activity. The activity of CYP1A2 in the vehicle-treated control was 28.1 ± 3.2 pmol/mg protein/min. CYP1A2 activity significantly decreased at 24 h after CLP, but this decrease was attenuated by Trolox (Fig. 4). CYP2B1 activity significantly decreased at 24 h after CLP. This decrease in enzyme activity was restored to normal levels by Trolox (Fig. 5). The CYP2E1 activity in the vehicle-treated control rats was 0.16 ± 0.1 nmol/mg protein/min. The CYP2E1 activity markedly decreased five times control values in CLP rats. This decrease was significantly attenuated by Trolox (Fig. 6).

DISCUSSION

Despite recent progress in antibiotic and critical care therapy, sepsis is still associated with a high mortality rate. Septic shock and sequential MODS correlate with poor outcome (Karima *et al.*, 1999), and septic shock is the most common cause of death in intensive care units (Parrillo *et al.*, 1990). This could be due to the fact that some of the subtle alterations in cellular functions that occur during the early stage of sepsis are not identified, leading to inadequate or delayed treatment of sepsis. Although the endotoxin model is highly reproducible and provides information on one aspect of sepsis, there is no evidence to suggest that such a mechanism operates in a clinically relevant condition of polymicrobial abdominal sepsis (Baveja *et al.*, 2002).

Because of its central role in metabolism and host defense mechanisms, the liver is believed to play a major role in the initiation of multiple organ failure, the most lethal complication in the clinical course of sepsis. In patients with sepsis and SIRS, the liver has two opposing roles: a source of inflammatory mediators and a target

organ for the effects of the inflammatory mediators (Szabo *et al.*, 2002). Microbes and their virulence factors enter the liver, where they first activate sinusoidal endothelial cells and Kupffer cells to produce proinflammatory mediators including ROS. These mediators cause not only microbial killing, but also structural and functional liver damage concerning mainly the parenchymal cells (Ring and Stremmel, 2000).

α-Tocopherol is well known as a strong natural antioxidant. One of the major functions of α-tocopherol is to inhibit lipid peroxidation (Marubayasch *et al.*, 1988). However, α-tocopherol is extremely lipophilic and taken up by cells slowly, *i.e.*, within days or weeks (Ingold *et al.*, 1987). Therefore, it is not an ideal therapeutic antioxidant, especially in an emergency setting. Trolox has been reported to scavenge peroxyl radicals from artificial systems better than its parent compound (Wu *et al.*, 1991). It has been observed that Trolox protects human myocytes and hepatocytes against oxyradicals generated *in situ* (Wu *et al.*, 1990). Furthermore, we observed that Trolox protected the liver cells against hypoxia/reoxygenation injury in isolated perfused rat livers (Lee and Cho, 1997). However, few of the studies cited have rigorously determined whether Trolox has antioxidant activity in an animal model.

In the present study, lipid peroxidation increased at 24 h after CLP. Interestingly, serum aminotransferases also increased at 24 h after CLP. Thus, our data show that a temporal association exists between increased lipid peroxidation and hepatic injury during sepsis. Trolox attenuated hepatic injury and lipid peroxidation, which suggests that it reduces oxidative stress during sepsis. Although temporally associated, it is not clear that microsomal lipid peroxidation and hepatocyte necrosis are causally linked. However, our findings may be indicative of hepatocyte oxidative stress that produces functional impairment of drug metabolism without directly contributing to hepatocyte necrosis. Membrane-associated functions such as activity of CYP isozymes may be more directly influenced by lipid peroxidation than by other aspects of liver injury (Lee *et al.*, 2000). In our data, total CYP concentrations were significantly decreased at 24 h after CLP, and this decrease was attenuated by Trolox. Alterations in the hepatic CYP-dependent drug-metabolizing enzyme system during sepsis is largely related to lipid peroxidation. Such a decrease in total CYP content suggests that the overall activity of CYP-dependent oxidations are likely to be similarly decreased. This decrease results from injury to the endoplasmic reticulum *via* the disruption of the membrane lipid environment, as well as the down-regulation of specific CYP isoforms. CYP requires NADPH-P450 reductase for electron transfer. NADPH-P450 reductase activity was also significantly decreased at 24 h after CLP This decrease was not attenuated by Trolox. This result suggests that ROS may more directly damage the CYP protein (heme protein), than the NADPH-P450 reductase protein (non-heme protein) in sepsis.

CYP1A1 and 1A2 are known as isozymes that exist in practically all mammalian species. Human hepatic microsome contains higher levels of CYP1A2 than CYP1A1, especially in cigarette smokers. CYP1A1 is detected in much higher levels than in non-smokers. Although subtle differences exist between two isozymes, the function of these isozymes is fairly well conserved across species. These isozymes play an important role in carcinogenesis as well as in xenobiotic metabolism (Daly, 1995). In the present study, both CYP1A1 and 1A2 activities were significantly decreased at 24 h after CLP. However, Trolox attenuated the decrease in CYP1A2 activity, but not in CYP1A1 activity. This result suggests that CYP1A2 is more sensitive to ROS produced during sepsis than is CYP1A1.

Isozymes belonging to the CYP2B subfamily have been studied extensively in many species. In the rat, CYP2B1/2B2 is known as the major phenobarbital-inducible CYP isozyme (Guengerich *et al.*, 1982). In the present study, the activity of CYP2B1 decreased at 24 h after CLP. Trolox restored CYP2B1 activity to the level of control rats. Thus, this result indicates that CYP2B1 is more sensitive to oxidative stress by ROS than other isozymes.

CYP2E1 is significant for its adaptive response to high blood ethanol levels, with a corresponding acceleration of ethanol metabolism, and is inducible by small organic molecules and pathophysiological states (Hong *et al.*, 1987). In addition, several studies were conducted to determine the relationship between induction of CYP2E1 and ROS (Nieto *et al.*, 2002; Parke and Sapota, 1996). In the present study, CYP2E1 activity was significantly decreased at 24 h after CLP, but this decrease was attenuated by Trolox. Although Trolox had a positive effect on CYP2E1 activity, the recovery of CYP2E1 activity was lower than that of other CYP isozymes. This result suggests that other factors (produced indirectly by cytokines generated during sepsis) than ROS may also be responsible for decrease in CYP2E1 activity.

The distinctive response of CYP isozymes to sepsis would be expected to result in variable effects on the intrinsic hepatic clearance of particular drugs in patients with sepsis. Our data indicates that Trolox ameliorates the hepatic drug metabolizing dysfunction that occurs during sepsis, and this effect is associated with decreased lipid peroxidation.

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