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Effect of Trolox C on Hypoxia/Reoxygenation-Induced Injury in Isolated Perfused Rat Liver

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Livers isolated from 18 hours fasted rats were subjected to N_2 hypoxia (for 45 min) followed by reoxygenation (for 45 min). The perfusion medium used was Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). Lactate and alanine were added as gluconeogenic and ureagenic substrates and Trolox C was also added to perfusate. Oxygen consumption, lactate dehydrogenase (LDH), alanine transaminase (ALT), total glutathione, oxidized glutathione, bile flow, glucose and urea were measured. After hypoxia oxygen consumption significantly dropped but Trolox C had no influence on this decrease. ALT and LDH were significantly increased by hypoxia/reoxygenation. This increase was markedly attenuated in the presence of Trolox C. The total glutathione and oxidized glutathione efflux increased following hypoxia, which were prevented by the treatment of Trolox C. Bile flow rate decreased following hypoxia/reoxygenation but did not continue to decrease in the reoxygenation phase by Trolox C. Following hypoxia/reoxygenation glucose and urea releases decreased. Trolox C had no influence on inhibition of glucose and urea production. These results suggest that Trolox C protected the liver cells against hypoxia/reoxygenation injury, yielding further evidence for a causative role of oxidative stress in this model.

Key words : Trolox C, Hypoxia/reoxygenation, Liver function, Oxidative stress

INTRODUCTION

With the increased application of new therapeutic strategies, like organ transplantation and cardiac reperfusion, the interest in the mechanisms underlying the cytotoxic effects observed following reperfusion of ischemia/hypoxic tissue has grown. In several studies reactive oxygen species have been implicated as the ultimate injuries species in reperfusion/reoxygenation damage of various organs (Granger *et al.*, 1981; Younes *et al.*, 1984; Das *et al.*, 1986; Brass *et al.*, 1991).

The liver seems to be particularly resistant to hypoxic as well as reperfusion injury, as long as anaerobic energy supply is maintained(Anundi *et al.*, 1987; Anundi and DeGroot, 1989). Depletion of glycogen or inhibition of glycolysis (Younes and Strubelt, 1988) makes the liver susceptible to tissue damage due to hypoxia solely or hypoxia and reoxygenation.

Vitamin E (α-tocopherol) is the best known natural antioxidant. However, vitamin E is extremely lipophilic and taken up by cells reactively slowly, *i.e.* within days or weeks (Ingold *et al.*, 1987). Therefore, it is not an ideal therapeutic antioxidant, especially in emergency setting. In 1974, Scott *et al.* (1974) synthesized Trolox C (or simply Trolox), hydrophilic analogue of vitamin E.

Trolox C was reported to protect dilinoleoyl phosphatidyl choline multilamellar liposomes (Doba *et al.*, 1985) and linoleic acid in SDS micelles (Barclay *et al.*, 1985) against peroxyl radicals generated *in situ.* Wu *et al.* (1990) observed that Trolox protects human myocytes, hepatocytes, and erythrocytes against *in situ* generated oxyradicals and illustrated, for the first time, that Trolox behaves mechanistically as an antioxidant in cultured human hepatocytes. However, none of the studies cited has rigorously determined whether Trolox has antioxidant activity in a clinically more relevant animal model of hepatic ischemia/reperfusion.

The aim of this study was to investigate the effect of water soluble vitamin E analogue Trolox C on hypoxia/reoxygenation in order to further evaluate the role of oxidative mechanisms in hypoxic liver injury.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats(250-300g) were used throughout. They had free access to a standard diet and tap water until the fasting was achieved by deprivation of feed but not of drinking water 18hr before surgery.

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Liver perfusion

Removal of the liver and its connection to a non-recirculating perfusion system was performed(Lee and Clemens, 1992). The perfusate was Krebs-Henseleit bicarbonate buffer, pH 7.4, saturated with 95% O_2-5% CO_2 maintained at 37°C. To induce hypoxia in the appropriate experiments, carbogen was replaced by a mixture of 95% N_2 and 5% CO_2 after equilibrium period. After 45 mins of hypoxia, reoxygenation was achieved by regassing with carbogen until the end of experiment, *i.e.* for 45 mins. Sodium taurocholate (26.7 g/L) was infused into the perfusate at a rate of 12 ml/hr to stimulate bile secretion. Livers were pre-perfused for at least 20 mins under basal conditions (Krebs buffer with no exogenous substrate) before measurements were started. After basal conditions, the perfusate was switched to one containing 4 mM lactate plus 10 mM alanine for the remainder of the experiment. Glucose was omitted from all perfusates to assess gluconeogenesis by rate of glucose appearance in the effluent as previously described (Ohtake and Clemens, 1991). Trolox was added to the perfusate at the end of the equilibration period to yield a final concentration of 0.5 mmol/L. At the end of experiments, the livers were weighed, frozen in liquid nitrogen and kept frozen until analysis.

Analytical and procedure

Oxygen concentration in the effluent perfusate was monitored continuously with a Clark-type oxygen electrode. Rates of O_2 uptake of whole livers were calculated from influent minus effluent O_2 concentration differences and the constant flow rates and were normalized for the wet weight of the liver (Matsumura and Thurman, 1983). Glucose in the effluent perfusate was determined using a glucose oxidase-peroxidase colorimetric assay (Sigma Chemical Co.). Gluconeogenesis was then calculated as [glucose] X flow/liver weight. Results for O_2 uptake and gluconeogenesis were then expressed as moles per gram per hour. Urea in the effluent perfusate using a diacetyl/monoxim colorimetic assay (Sigma Chemical Co.). The activities of alanine transaminase in the perfusate was determined by standard spectrophotometric procedures using Sigma kit. Total glutathione was determined according to Brehe and Burch (1978); oxidized glutathione was estimated by the same procedure after blocking reduced glutathione with 2-vinylpyridine (Griffith, 1980).

Statistics

All data were expressed as means±S.E.M. The difference between two means was checked with Dunnet's *t*-test in the case of multiple comparisons or with Student's *t*-test in the case of simple comparisons. The limit of significance was $p<0.05$ in all cases.

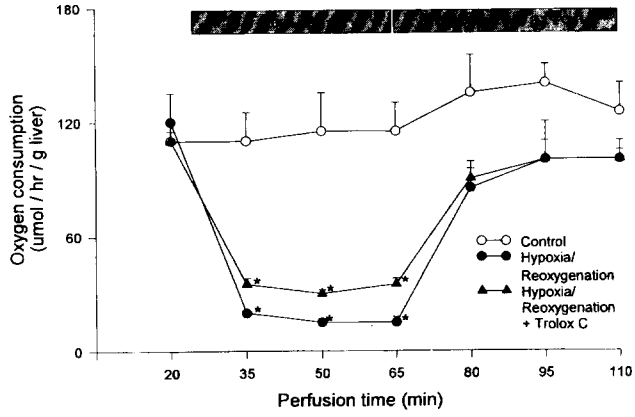

Fig. 1. Time-dependent oxygen consumption by isolated perfused livers. Values are means±S.E.M. for 7 to 10 rats per group. *=p<0.05 vs control.

RESULTS

Under normoxic conditions with oxygen partial pressure around 600 mmHg, oxygen consumption by isolated perfused liver was between 100 and 150 μmol h^{-1} g $liver^{-1}$ over the whole perfusion period (Fig. 1). When carbogen was replaced by 95% N_2-5% CO_2 oxygen partial pressure fell to 100 mmHg. As a consequence, oxygen consumption dropped abruptly down to a minimum of 15 μmol h^{-1} g $liver^{-1}$ at the end of the hypoxic period (i.e. after 45 mins). Following reoxygenation, oxygen consumption rose again but only reached 80% of the value before hypoxia. Trolox C had no significant effect on the course of oxygen consumption during experiment.

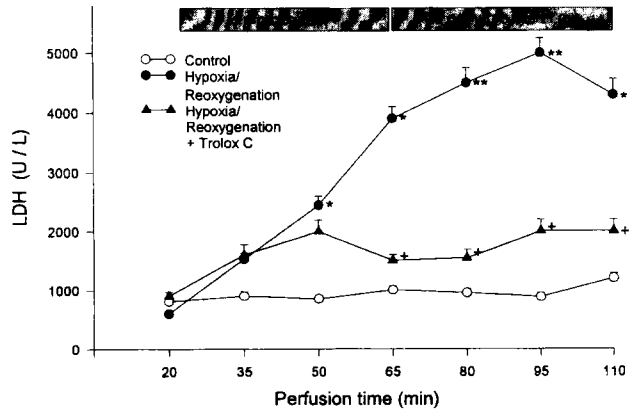

Fig. 2. Time-dependent release of LDH by isolated perfused livers. Values are means±S.E.M. for 7 to 10 rats per group. *=p<0.05, **=p<0.01 vs control. +=p<0.05 vs hypoxia/reoxygenation.

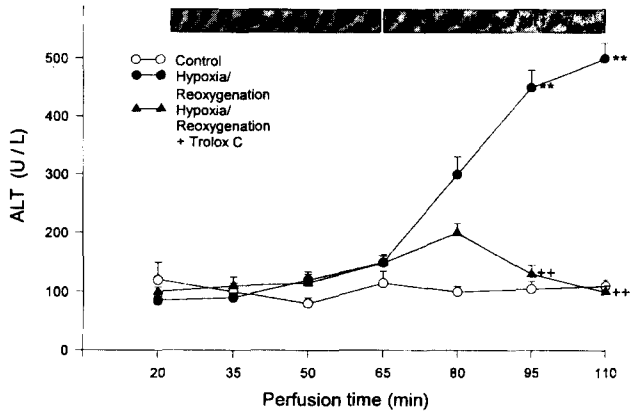

Fig. 3. Time-dependent release of ALT by isolated perfused livers. Values are means±S.E.M. for 7 to 10 rats per group. **=p<0.01 vs control. ++=p<0.01 vs hypoxia/reoxygenation.

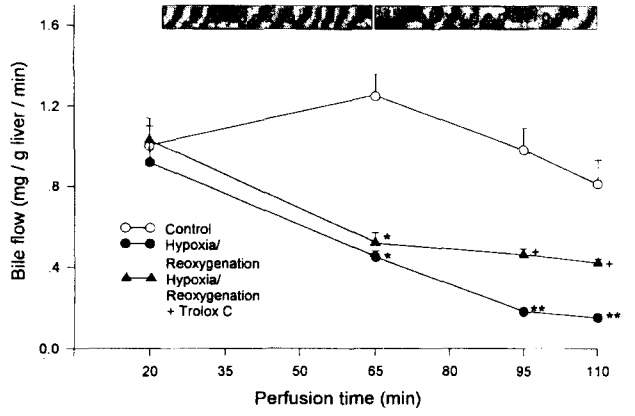

Fig. 4. Time-dependent bile flow rate by isolated perfused livers. Values are means±S.E.M. for 7 to 10 rats per group. *=p<0.05, **=p<0.01 vs control. +=p<0.05 vs hypoxia/reoxygenation.

Damage to the isolated perfused livers was estimated by measuring the efflux of the cytosolic enzymes lactate dehydrogenase (LDH) and alanine transaminase (ALT) (Fig. 2 and Fig. 3). When livers from rats that had been fasted for 18hr were perfused with carbogen, LDH and ALT releases were minimal. No change was observed in ALT release but LDH release started to increase a following hypoxia. Furthermore, reoxygenation resulted in a strong sustained increase of ALT and LDH releases into the perfusate. These increases were markedly attenuated in the presence of Trolox C.

The total glutathione efflux, other index of oxidative stress, was dramatically enhanced at the end of hypoxic period that dropped rapidly after reoxygenation, which was prevented by the treatment of Trolox C (Table I). The GSSG efflux showed similar trends of total glutathione efflux. Trolox C also inhibited this increase during the reoxygenation period.

Bile flow showed little variation with perfusion time in control experiments without hypoxia and reoxygenation (Fig. 4). Hypoxia led to a strong decline in bile flow rate which continued also following reoxygenation, reaching finally 20% of the original rate. In the presence of Trolox C, on the other hand, bile flow rate declined in the hypoxic phase to the same extent as in the experiments without antioxidant, but did not continue to decrease in the reoxygenation phase.

In the absence of exogenous substrate, isolated perfused livers produced glucose at a rate of <5 μmol g^{-1}

Table I. Changes in concentration of glutathione after hypoxia/reoxygenation

Group	Perfusion time (min)	Total glutathione (μmol/L)	Oxidized glutathione (μmol/L)
Control	20	6.5±1.4	3.4±0.4
	35	9.1±1.2	4.0±0.2
	65	7.2±1.1	4.6±0.4
	80	6.1±1.3	3.7±0.6
	110	5.4±1.4	2.8±0.5
Hypoxia+Reoxygenation	20	7.1±1.1	4.0±0.5
	35	20.1±1.2*	11.4±0.1*
	65	26.3±2.1**	13.4±0.1**
	80	12.1±2.3	6.0±1.7
	110	10.4±3.4	4.9±0.6
Hypoxia+Reoxygenation+Trolox C	20	5.1±1.1	3.2±0.3
	35	6.3±0.7+	3.8±0.1+
	65	8.1±0.9++	4.2±0.1+
	80	5.2±1.6	4.4±0.6
	110	5.6±1.2	3.8±0.7

Values given are means and their standard errors(means±S.E., n=7-10).
After a 20 min equilibration period, hypoxia was induced from 20 to 65 min followed by reoxygenation from 65 to 110 min.
*=p<0.05, **=p<0.01 vs control. +=p<0.05, ++=p<0.01 vs hypoxia/reoxygenation.

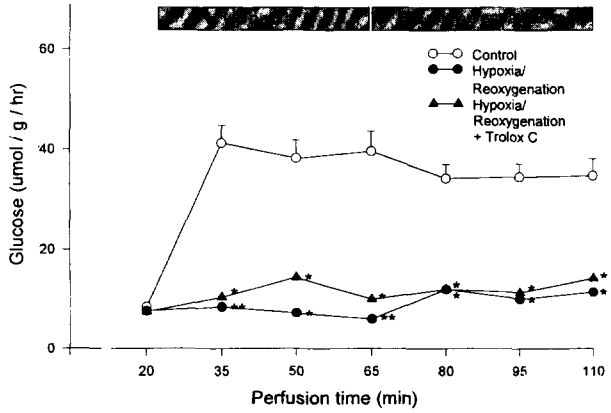

Fig. 5. Time-dependent glucose release by isolated perfused livers. Values are means±S.E.M. for 7 to 10 rats per group. *=p<0.05, **=p<0.01 vs control.

h^{-1} indicating substantial glycogen depletion as would be expected in fasted rats. The addition of 5 mM lactate plus 10 mM alanine resulted in approximately ninefold increase in glucose release. In sharp contrast, no increased release of glucose was evident following hypoxia and reoxygenation. Still, low value of glucose release was observed with Trolox C (Fig. 5).

Similar to glucose release, in the basal perfusate, isolated perfused livers produced minimal urea nitrogen but fourfold increase in urea release following substrate addition. No increase in urea release was observed following hypoxia but a tendency to increase this value was observed during reoxygenation. Trolox C had no influence on inhibition of urea production (Fig. 6).

DISCUSSION

Of 1,500 autopsies performed on patients with heart failure, 13% had pericentral liver damage (De La Monte *et al.*, 1984). Because pericentral regions of the liver lobule have lower oxygen tension, this observation is indicative of hypoxic damage due to diminished hepatic blood flow. Several reports also suggest that hypoxia plays an important role in alcohol-induced liver damage (Israel *et al.*, 1975 ; Yuki and Thurman, 1980). In addition, hypoxia may be particularly important in organ transplantation.

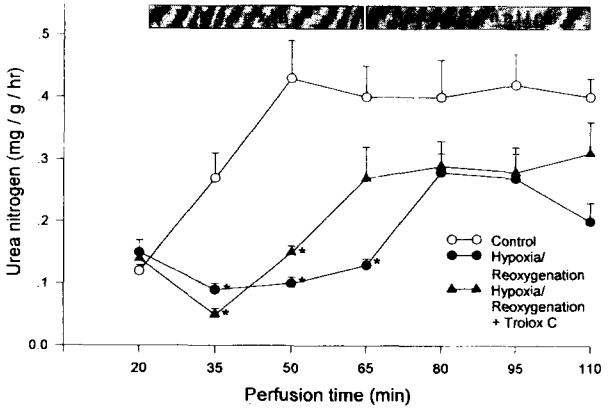

Fig. 6. Time-dependent release of urea nitrogen by isolated perfused livers. Values are means±S.E.M. for 7 to 10 rats per group. *=p<0.05 vs control.

Recent pathophysiological studies of ischemic injury suggest that part of the injury also occurs during the period of reperfusion when reactive oxygen metabolites are generated (McCord, 1985). We have previously reported that α-tocopherol pretreatment substantially attenuated increases in hepatic lipid peroxidation during ischemia/reperfusion. In the present study we have extended these findings to water soluble vitamin E analogue Trolox C. Perfusion of livers with nitrogen-saturated perfusate is a convenient and useful model to study hypoxic damage (Yuki and Thurman, 1980).

Our study clearly showed cell damage (LDH, ALT, total glutathione, GSSG release) occurred after prolonged hypoxia (45 mins) in livers from rats which were deprived of food 18hr prior to surgery. Under these conditions, hepatic glycogen is nearly total depleted (Younes *et al.*, 1988).

Bile excretion has been observed to be suppressed in ischemia and restored up recirculation. These changes in bile secretion have been ascribed to depression of cellular level of ATP. A correlation between the bile flow rate and the cellular level of ATP was reported by Slater and Delaney more than 10 years ago (Slater and Delaney, 1970). Kamiike *et al.* (1985) has shown that extent of hepatic injury can be assessed simply by monitoring the bile flow rate, which was felt to reflect the cellular level of ATP. In our studies impairment of bile excretion occurred following hypoxia.

Trolox C protected isloated perfused rat liver against hypoxic injury and ameliolated depressed bile secretion. In recent studies with cultured rat hepatocytes (Wu *et al.*, 1991) or with single-path perfused rat liver (Videla, 1991), Trolox C also proved to be protective against reperfusion injury. These observations provide a strong indication for an involvement of oxidative stress in hypoxia/or reperfusion hepatic injury.

We attempted to measure glucose release and urea production to assess liver function following hypoxia/reoxygenation. Under basal conditions, rates of glucose release and urea production were minimal and constant regardless of hypoxia/reoxygenation (data not shown). The addition of lactate and alanine resulted in not only increases in glucose release but also in urea production. As expected, hypoxic livers decreases glucose release and urea production. But unfortunately, Trolox C did not inhibit the decreases of glucose releases and urea productions.

In conclusion, the inhibition of hypoxia/reoxygenation injury in isolated perfused rat livers by Trolox C strongly substantiates a role for oxidative stress in this type of tissue injury. Trolox C may be of therapeutic value especially in emergency situations due to its water solubility and high efficacy as free radical scavenger.

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