Protective Role of α -Tocopherol Acetate against Thioacetamide-Induced Mitochondrial Dysfunction

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Summary It was reported earlier that administration of an acute dose of thioacetamide to rats produced changes in the rate of respiration, phosphate to oxygen ratio, and respiratory control ratio when either glutamate + malate or succinate were used as substrates (Padma and Setty, *J. Clin. Biochem. Nutr.*, **22**, 113–123). A significant decrease in the activities of electron transport chain enzymes was also observed. In the present study we report that administration of vitamin E prior to thioacetamide administration provided significant protection against the mitochondrial dysfunctions induced by thioacetamide. We speculate that the protective effect of vitamin E may be due to the ability of this vitamin to suppress elevated lipid peroxide levels in rats.

Key Words: mitochondrial dysfunction, lipid peroxidation, vitamin E, oxidative phosphorylation, hepatotoxin

Mitochondria carry out a very important function of the cell, i.e., oxidative phosphorylation. Interaction of mitochondria with cytotoxic compounds causes mitochondrial dysfunction and leads to cell death. Administration of compounds such as thioacetamide [1], doxorubicin [2], cisplatin [3], galactosamine [4, 5], and ethanol [6] have been shown to cause disturbances in mitochondrial function accompanied by increased lipid peroxidation. Prior treatment with antioxidants like vitamin E [2, 4, 7] or selenium [3] protected against the cytotoxicity caused by these hepatotoxins.

Thioacetamide is one of the various hepatotoxins used to induce acute liver failure in animals [8]. This agent is known to induce necrosis [9], cirrhosis, and tumors [10, 11], depending on the dosage and duration of treatment. Chronic

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Abbreviations used: ADP, adenosine diphosphate; 2,4-DNP, 2,4-dinitrophenol; DCPIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate; P/O ratio, phosphate to oxygen ratio; RCR, respiratory control ratio.

administration of thioacetamide to rats has been reported to cause cirrhosis of the liver accompanied by decreased mitochondrial respiration [12].

In a previous study, we reported that administration of an acute dose of thioacetamide produced alterations in mitochondrial function. Prior treatment with an aqueous extract of *Phyllanthus fraternus*, a medicinal plant, protected against the mitochondrial dysfunction induced by thioacetamide [1].

Vitamin E, a major lipid soluble antioxidant of membranes [13], is present in both inner and outer mitochondrial membranes [14]. Oxidative stress destroys vitamin E and causes its depletion within minutes [15]. In vitamin E-deficient rabbits, the phosphate to oxygen (P/O) ratio with succinate as substrate decreased from 1.7 to 1.1 [16]. α -Tocopherol has been shown to protect against doxorubicin-induced lipid peroxidation in mitochondria [2] and also against hepatotoxicity induced by ethanol and galactosamine [4]. In the present study, the protective effect of α -tocopherol against thioacetamide-induced mitochondrial dysfunction is reported.

MATERIALS AND METHODS

Chemicals. Adenosine diphosphate (ADP), 2,4-dinitrophenol (2,4-DNP), cytochrome *c*, adenosine triphosphate, and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from local firms.

Treatment of experimental animals. Male Wistar rats (100–120 g) were used for the present study, and were divided into four groups: 1) the control group, which received 0.2 ml of saline intraperitoneally; 2) the thioacetamide group, which received thioacetamide (25 mg/100 g body weight/in 0.2 ml saline), intraperitoneally for four days, and was sacrificed 24 h after the last dose; 3) the vitamin E group, which received 20 IU of vitamin E orally per day per rat for 20 days. This treatment raised vitamin E levels by 50% (measured by HPLC); 4) the vitamin E + thioacetamide group, which received vitamin E as described above and then thioacetamide (25 mg/100 g body weight in 0.2 ml saline) given for four days and was sacrificed 24 h after the last dose.

Isolation of mitochondria. Mitochondria from liver were isolated by differential centrifugation according to the method of Lawrence and Davies [17]. Protein was measured by the biuret method [18] with bovine serum albumin used as the standard.

Assay of oxidative phosphorylation. Polarographic measurements of oxygen consumption were made with a Clark-type oxygen electrode in a Gilson 5/6 oxygraph. Rate of respiration, respiratory control ratio (RCR), and P/O ratio were calculated according to Estabrook [19].

ATPase assay was carried out according to the method described earlier [20]. The inorganic phosphate released was estimated by the Fiske-Subbarow method [21].

Assay of enzymes. Spectrophotometric assays of the respiratory enzymes were conducted with a Shimadzu 160A spectrophotometer.

NADH dehydrogenase activity was measured at 420 nm with potassium ferricyanide used as an electron acceptor [22]. Succinate dehydrogenase was assayed with phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCPIP) used as electron acceptors. The reduction of DCPIP was followed at 600 nm [23]. Succinate-cytochrome *c* reductase activity was measured by following the reduction of ferricytochrome *c* at 550 nm [24]. Cytochrome oxidase activity was measured by following the rate of oxidation of ferrocytochrome *c* at 550 nm [25]. The activity of these enzymes (except cytochrome oxidase) was expressed as nmol reduced per min per mg protein. Cytochrome oxidase activity was expressed as nmol of cytochrome *c* oxidized per min per mg protein. The concentrations of cytochromes *aa*₃, *b*, and *c* were determined from a difference spectrum (dithionite reduced minus oxidized) recorded at room temperature with a Hitachi double beam spectrophotometer [26]. Lipid peroxide level was determined in liver homogenates and mitochondria of control and treated animals according to the method of Ohkawa *et al.* [27]. 1,1,3,3-Tetramethoxypropane was used as an external standard.

Mitochondrial lipids were separated by the procedure of Bligh and Dyer [28]. Phospholipids were separated by thin-layer chromatography using chloroform : methanol : water (65 : 25 : 4) as solvent. The individual phospholipids were digested with 60% perchloric acid, and the inorganic phosphate was estimated by the Fiske-Subbarow method [21].

Statistical analyses was conducted by Student's *t*-test, and a *p* value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

The effect of thioacetamide administration on mitochondrial function and the protective effect of vitamin E on thioacetamide-induced toxicity were studied.

Studies on NADH oxidase gives information on the ability of the electron transport chain to transfer electrons through three sites. State-3 respiration decreased by 59%, whereas RCR and P/O ratio were decreased by 80 and 99%, respectively, in the thioacetamide-treated rats. Administration of vitamin E prior to thioacetamide relieved the inhibition of state-3 respiration completely, and RCR and P/O ratio were relieved by 76 and 94%, respectively. Administration of vitamin E alone did not have any significant effect on rate of respiration, RCR or P/O ratio (Table 1).

Succinate oxidase, which gives information on the rate of electron transfer through site II and site III, was decreased by 47% in thioacetamide-treated rats; whereas RCR and P/O ratio were decreased by 73 and 49%, respectively. Administration of vitamin E prior to thioacetamide relieved the reduction of state-3 respiration, RCR, and P/O ratio by 81, 80, and 88%, respectively (Table 1).

Table 1. Effect of administration of thioacetamide with or without pretreatment with vitamin E using glutamate+malate or succinate as substrates.

Group	State-3 respiration	RCR	P/O
With glutamate+malate			
Control	100±12	100±4.5	100±2.5
Thioacetamide	41±4*	20±1**	1±0.1**
Vitamin E	96±12	90±2	97±1
Vitamin E+thioacetamide	104±3	81±4	94±1
With succinate			
Control	100±7	100±1	100±2
Thioacetamide	53±7*	27±1**	51±1*
Vitamin E	95±5	108±2	99±1
Vitamin E+thioacetamide	91±14	85±1	94±1

All the values are expressed relative to the control, which was taken as 100. The control values for state-3 respiration, RCR, and P/O ratio were 73 ± 9 , 5.2 ± 0.24 , and 3.03 ± 0.02 respectively, when glutamate+malate was used as substrates. The state-3 respiration, RCR, and P/O ratio were 80 ± 6 , 4.06 ± 0.05 , and 1.96 ± 0.04 respectively, when succinate was used as substrate. Values are mean±SD from eight animals. * $p<0.005$; ** $p<0.001$ vs. corresponding control.

Decreased RCR and P/O values in thioacetamide-treated rats indicate damage to the mitochondrial membrane, which thereby renders the membrane leaky to ions and biomolecules and consequently leads to uncoupling of mitochondria. Increased permeability of the mitochondrial membrane has been shown to cause uncoupling of oxidative phosphorylation [29]. When vitamin E, which acts as a biological antioxidant and preserves membrane integrity [30], was administered prior to thioacetamide treatment, the vitamin E protected against the reduction of RCR and P/O ratios.

The finding that the mitochondria were uncoupled by thioacetamide treatment was also confirmed by studies on ATPase activity. Thioacetamide treatment significantly increased the ATPase activity both in the presence and absence of 2,4-DNP. ATPase stimulation with 2,4-DNP was significantly decreased in thioacetamide-treated rats as compared with that of the control (32% for thioacetamide vs. 139% for the control). Vitamin E restored the 2,4-DNP stimulation that was knocked out by thioacetamide close to the control level (94% compared to 139% for the control, Table 2). Tightly coupled mitochondria show very little ATPase activity, and this activity can be elicited by the addition of an uncoupler. On the other hand, uncoupled mitochondria show very high ATPase activity and cannot be stimulated further with an uncoupler like 2,4-DNP.

NADH dehydrogenase, which gives electrons to the electron transport chain at the site-I level, was decreased in activity by 61% in thioacetamide-treated rats. The observed decrease in such activity may limit the rate of electron flow through site I and effect energy conservation in this region of the respiratory chain. Administration of vitamin E prior to thioacetamide relieved the inhibition of NADH dehydrogenase by 72% (Table 3).

Table 2. Effect of administration of thioacetamide with or without pretreatment with vitamin E on ATPase activity of liver mitochondria in the presence and absence of DNP.

Group	-DNP	+DNP	% Stimulation with DNP
Control	1.53±0.25	3.66±0.14	139
Thioacetamide	5.34±0.15**	7.06±0.24**	32
Vitamin E	1.60±0.12	3.26±0.21	103
Vitamin E+thioacetamide	1.73±0.35	3.36±0.2	94

Activity of the enzyme is expressed as μg phosphate released per min per mg protein. Results are expressed as mean $\pm$ SD of six animals. ** $p < 0.001$ vs. corresponding control.

Table 3. Effect of administration of thioacetamide with or without pretreatment with vitamin E on enzymes of the respiratory chain.

Group	NADH dehydrogenase	Succinate dehydrogenase	Succinate-cytochrome <i>c</i> reductase	Cytochrome oxidase
Control	2,250±50	159±9	68±16	1,469±21
Thioacetamide	878±157*	161±18	31±4**	931±55*
Vitamin E	2,120±75	165±8	59±3	1,525±25
Vitamin E+thioacetamide	1,875±100	156±8	52±4	1,200±15

Values are the mean $\pm$ SD for four animals. Activities of the enzymes were calculated per min per mg protein as described in Materials and Methods. * $p < 0.02$; ** $p < 0.001$ vs. corresponding control.

Table 4. Effect of administration of thioacetamide with or without pretreatment with vitamin E on cytochrome contents of rat liver mitochondria.

Group	Cytochrome concentration		
	<i>aa</i> ₃	<i>b</i>	<i>c</i>
Control	100±4	100±1	100±1
Thioacetamide	76±1*	78±1	88±1
Vitamin E	107±1	85±1	83±1
Vitamin E+thioacetamide	87±2	96±1	93±1

Values are given as percent of the control, which was taken as 100 and are the mean $\pm$ SD of eight animals. The control values for cytochrome *aa*₃, *b*, and *c* were 0.243±0.009, 0.27±0.001, and 0.375±0.002 nmol per mg protein, respectively. * $p < 0.05$ vs. corresponding control.

Succinate-cytochrome *c* reductase gives information about site II of the electron transport chain. It was decreased by 54% in thioacetamide-treated rats. Cytochrome oxidase, the terminal enzyme of the respiratory chain, was also decreased by 37% in the thioacetamide-treated animals. Pretreatment with vitamin E protected against the inhibition of succinate-cytochrome *c* reductase and cytochrome oxidase by 56 and 51%, respectively. Succinate dehydrogenase was unaffected by either thioacetamide alone or thioacetamide in the presence of high levels of vitamin E (Table 3).

Table 5. Effect of administration of thioacetamide with or without pretreatment with vitamin E on lipid peroxide levels in liver homogenates and mitochondria.

Group	Lipid peroxide level	
	Liver homogenate	Mitochondria
Control	130 $\pm$ 10	86 $\pm$ 12
Thioacetamide	210 $\pm$ 22**	113 $\pm$ 9*
Vitamin E	128 $\pm$ 12	80 $\pm$ 5
Vitamin E + thioacetamide	150 $\pm$ 12	93 $\pm$ 5

Lipid peroxide level is expressed as nmol malondialdehyde formed per 100 mg protein. Results are the mean $\pm$ SD of eight animals. * p < 0.05; ** p < 0.005 vs. corresponding control.

Table 6. Effect of administration of thioacetamide with or without pretreatment with vitamin E on phospholipid composition of rat liver mitochondria.

Group	Phosphatidyl choline	Phosphatidyl ethanolamine	Cardiolipin
Control	100 $\pm$ 12	100 $\pm$ 9	100 $\pm$ 6
Thioacetamide	58 $\pm$ 4**	72 $\pm$ 3*	56 $\pm$ 3**
Vitamin E	98 $\pm$ 6	91 $\pm$ 1	92 $\pm$ 1
Vitamin E + thioacetamide	84 $\pm$ 1	86 $\pm$ 1	107 $\pm$ 1

Results are given as a percent of the control and are the mean $\pm$ SD of six animals. The control values for phosphatidyl choline, phosphatidyl ethanolamine, and cardiolipin were 21.6 $\pm$ 2.5, 16.7 $\pm$ 1.5, and 13.3 $\pm$ 0.7 μ g phospholipid phosphorus per g tissue, respectively. * p < 0.01; ** p < 0.005; vs. corresponding control.

The concentrations of cytochrome aa_3 and b decreased by 24 and 22% respectively, in thioacetamide-treated rats, whereas the decrease in the concentration of cytochrome c was insignificant (12%) (Table 4). The decrease in the level of cytochrome aa_3 and b induced by thioacetamide was relieved by 46 and 82% respectively, by pretreatment with vitamin E. Respiratory process involves the transport of electrons through cytochromes to molecular oxygen, and any change in the cytochrome concentrations may thus affect the transport of electrons via the respiratory chain and alter the energy production of mitochondria. Earlier studies demonstrated that lipid peroxidation caused damage to cytochromes aa_3 , b , and c in isolated electron transport chains [16].

The lipid peroxide level, an index of membrane damage, increased by 61% in liver homogenates and by 31% in mitochondria of thioacetamide-treated rats. Administration of vitamin E alone did not show any significant effect on the lipid peroxide level; but when thioacetamide was administered in the presence of a high level of vitamin E, the elevation in lipid peroxide level was brought down from 61 to 15% in homogenate and from 31 to 8% in mitochondria (Table 5).

Upon thioacetamide treatment, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin decreased by 42, 28, and 44%, respectively, as compared with control values. Administration of vitamin E alone did not affect any of these

levels. Vitamin E reversed the effects of thioacetamide on the phospholipid composition and raised the levels close to control values. In vitamin E-pretreated rats the reduction in phosphatidylcholine, phosphatidylethanolamine, and cardiolipin levels was relieved by 62, 50, and 115%, respectively (Table 6). Cardiolipin plays a key role in the activity of several enzymes localized in the inner mitochondrial membrane [31], including enzymes at site I and site III of the respiratory chain; whereas enzymes at site II lack the requirement for cardiolipin [32]. Arai *et al.* [33] reported that phosphatidylcholine and phosphatidylethanolamine are essential for the normal functioning of cytochrome oxidase in ethanol-fed baboons.

Our results show that acute administration of thioacetamide to rats leads to mitochondrial dysfunction. Dietary supplementation of vitamin E prior to thioacetamide treatment prevented the toxicity induced by thioacetamide and lessened the reduction in values of all the mitochondrial parameters studied. The induction of lipid peroxidation *in vitro* in rat liver mitochondria by Fe^{2+} /ascorbate and NADPH/ADP/ Fe^{2+} caused an 80% decrease in α -tocopherol concentrations accompanied by an increase in lipid peroxide levels [34]. Thomas *et al.* [35] showed that vitamin E deficiency led to reduced stability of mitochondrial membranes and decreased the rate of oxygen utilization with both NADH- and FADH_2 -linked substrates.

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