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Lipid peroxidation is believed to be an important process in the development of cellular injury. In addition to the degradation of biological membranes, increasing evidence indicates that lipid peroxidation can alter the structure and function of DNA.

The present study examines the peroxidation of nuclear membranes and the capacity of glutathione to inhibit this process. Research indicates that glutathione protects isolated rat liver nuclei against both ascorbate and NADPH induced peroxidation. Evidence suggests that a glutathione dependent peroxidase associated with the nuclear membrane is required for this protection. Inhibition and substrate specificity requirements show that this peroxidase is a glutathione transferase. Partially purified nuclear glutathione transferase

lipid hydroperoxides. Since lipid hydroperoxides can catalyze the propagation of lipid peroxidation, the reduction of these compounds in biological membranes should contribute to the inhibition of lipid peroxidation.

Vitamin E has also been shown to be an effective inhibitor of lipid peroxidation *in vitro*. In experiments conducted with isolated nuclei supplemented with vitamin E, vitamin E protected against lipid peroxidation only when vitamin E was present above a certain critical level. If vitamin E levels were below this threshold value, peroxidation was not inhibited. The addition of glutathione reduced the threshold levels of vitamin E required to protect against lipid peroxidation. These results show that vitamin E and glutathione act synergistically to inhibit nuclear lipid peroxidation.

Research has shown that nuclear membranes contain pores which allow the free diffusion of small water soluble metabolites between the nucleus and cytoplasm. Experiments were conducted to determine the levels of glutathione in the nucleus. Rat kidney cell nuclei were isolated by nonaqueous isolation techniques to minimize the loss of glutathione during isolation. Results suggest that with normal cellular glutathione levels, the concentration of glutathione in the nucleus is similar to that found in the cytoplasm. Administration of buthionine sulfoximine to rats reduced cellular glutathione levels in the kidney. However, nuclear glutathione levels were

reduced to a much greater extent than cytosolic levels.

STUDIES ON THE GLUTATHIONE DEPENDENT PROTECTION OF THE CELL NUCLEUS AGAINST LIPID PEROXIDATION

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INTRODUCTION

Lipid peroxidation is the oxidative degradation of polyunsaturated lipids. Although the chemistry behind the peroxidation of unsaturated lipids is well characterized, the biological and pathological significance of these events remain in doubt. Lipid peroxidation has been associated with mechanisms of aging, the toxicity of certain xenobiotics, and in some phases of atherosclerosis (1). Reports also indicate that the peroxidation of polyunsaturated lipids can alter the structure of biological membranes (2) and lead to enzyme inactivation (3,4). Although all membrane proteins can be modified and fragmented by radicals produced during lipid peroxidation (5), reports indicate that enzymes possessing sulfhydryl groups are especially sensitive to inactivation by peroxidized unsaturated fatty acids (6). Lipid peroxidation, therefore, is potentially disruptive to normal cellular function and as such may be an important pathologic mechanism.

The cell possesses defensive mechanisms which can protect the polyunsaturated lipids of cellular membranes against oxidation. Vitamin E is a lipophilic compound which functions as an important antioxidant *in vivo*. The exact chemical mechanism of this compound is still

unknown, but vitamin E is believed to react with lipid peroxy radicals and thereby inhibit the propagation phase of lipid peroxidation (7).

In addition to vitamin E, other endogenous compounds have been implicated in the role of protecting cellular lipids against peroxidation. Ascorbate can function as a free radical scavenger in aqueous solutions (8), and in so doing, may be involved in the inhibition of the initiation of lipid peroxidation by water soluble initiators. Studies also suggest that ascorbate acts synergistically with vitamin E to inhibit lipid peroxidation (8,9). Research conducted by Scarpa et al. (10) shows that ascorbate can regenerate vitamin E incorporated in liposomes. The regeneration of vitamin E by ascorbate may be important in extending the ability of vitamin E to inhibit the propagation phase of lipid peroxidation.

The nonpolar compound β-carotene may also function as an important antioxidant in vivo. Burton and Ingold (11) found that β-carotene reacts with peroxy radicals at low partial pressures of oxygen. These researchers concluded that β-carotene may function in a similar manner as vitamin E in inhibiting the propagation phase of lipid peroxidation in those tissues exposed to low partial pressures of oxygen. Glutathione (GSH) is also believed to function as a cellular antioxidant, but the mechanism of its action is for the most part unknown.

The content of antioxidants in cellular membranes may

determine in part the relative susceptibility of these membranes to lipid peroxidation. Also, the relative content of such agents as proteins, peroxidation insensitive lipid and cholesterol may determine rates of lipid peroxidation in biological membranes. In research conducted by Mowri et al. (12), the addition of cholesterol and peroxidation-insensitive lipid to liposomes suppressed ferrous-ascorbate induced lipid peroxidation. Although cholesterol itself may be oxidized, research indicates that it is more resistant than polyunsaturated fatty acids to peroxidation (13) and therefore may inhibit lipid peroxidation. Thus, numerous factors determine the susceptibility of biological membranes to lipid peroxidation.

GLUTATHIONE DEPLETION AND LIPID PEROXIDATION

Several experiments conducted both *in vivo* and *in vitro* stress the importance of GSH in the inhibition of lipid peroxidation. In a study utilizing isolated rat hepatocytes, incubations with a series of compounds depleted intracellular GSH and induced lipid peroxidation and cell lysis (14). Increased malondialdehyde (MDA) levels were detected about thirty minutes prior to cell lysis. Both lipid peroxidation and cell lysis could be prevented by including either GSH precursor amino acids (cysteine, methionine) or α-tocopherol in the incubation media. The specific effects of 0.2 mM chloroacetamide on intracellular GSH levels were examined in this study by Anundi et al. (14). Following treatment of hepatocytes with this compound, a rapid rise in MDA levels began after GSH levels were reduced to approximately 10 percent of initial values. When methionine was included along with chloroacetamide in the incubation media, GSH levels were depleted to only 30 percent of initial values and no increases in MDA production or cell lysis were detected.

Similar results were obtained by Casini et al. (15) *in vivo*. These researchers studied the mechanism of bromobenzene, diethylmaleate (DEM), and iodobenzene hepatotoxicity in starved mice. Administration of these compounds by gastric intubation depleted hepatic GSH and induced lipid peroxidation and liver necrosis. A series

of experiments using varying doses of these xenobiotics revealed that GSH must be decreased below a critical value in order for lipid peroxidation to occur. When quantities of GSH were below a threshold value of (3.5 - 2.5 nmol/mg protein) or (14.8 - 10.6% of controls), lipid peroxidation and liver necrosis were observed. However, no lipid peroxidation or necrosis was seen if GSH levels were above these values. The administration of Trolox C, a vitamin E homolog, after bromobenzene treatment prevented both lipid peroxidation and necrosis. Experiments with acetaminophen (16) and ethanol (17) also indicate that GSH depletion precedes and may be required for the production of lipid peroxidation.

Investigations with starved mice treated with acetaminophen provide further support for the involvement of a GSH threshold in the onset of lipid peroxidation (18). Pooled results with rats injected with various doses of acetaminophen indicate that GSH levels must be depleted below 30 percent of controls before high quantities of ethane were expired.

Although there is good evidence that GSH depletion precedes and indeed may be required for lipid peroxidation to occur, GSH depletion alone does not appear to be sufficient to induce lipid peroxidation. Phorone reduced GSH levels in isolated rat hepatocytes to approximately 20 percent of controls, but MDA levels were the same in both untreated and treated hepatocytes (19). In a similar

manner, isolated rat hepatocytes incubated with the glutathione reductase inhibitor 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) exhibited GSH levels about 35 percent of initial levels (20). This treatment, however, was not associated with increased lipid peroxidation or cell lysis.

The exposure of isolated hepatocytes to the combined effects of 75 uM BCNU and 100 uM adriamycin reduced GSH levels to 5 percent of initial values and was associated with elevated MDA levels and cell death. Glutathione levels were depleted to a similar extent by treatment with both 75 uM BCNU and 340 uM DEM, but these conditions produced significantly lower MDA levels and increased cell viability over those seen with BCNU and adriamycin. Adriamycin, unlike DEM, is believed to generate activated oxygen species in the cell. This additional oxidative stress induced by adriamycin may promote lipid peroxidation following GSH depletion (20).

Analogous conclusions were reached by Younes et al. (21) with rats injected with the GSH depleting compound phorone. Injection of phorone resulted in only slight increases in ethane exhalation over the next twenty four hours. Administration of ferrous iron in conjunction with phorone, however, significantly elevated levels of expired ethane over those found in rats treated only with ferrous iron or phorone. In this study, iron was proposed to function in much the same manner as adriamycin did in the

previous investigation--that is, by initiating lipid peroxidation. Thus, it appears that GSH depletion below a certain critical threshold predisposes a cell to lipid peroxidation. Once this occurs, the expression of lipid peroxidation requires additional initiation factors.

The role of GSH in the inhibition of lipid peroxidation has also been examined with rat liver homogenates. In experiments conducted by Younes and Siegers (22), GSH depleting agents were administered to phenobarbital induced rats. Post mitochondrial (9000 x g supernatants) liver homogenates prepared from these animals were then incubated with a NADPH regenerating system under oxygen. Homogenates depleted of GSH produced higher MDA levels than those containing normal levels of GSH. Results from these experiments indicate that GSH must be decreased to 20 percent of initial values in order for lipid peroxidation to take place in this system. The addition of the antioxidant diethyl dithiocarbamate to homogenates inhibited MDA production but no effect was seen with scavengers of excited oxygen species such as superoxide dismutase or catalase (22).

GLUTATHIONE DEPENDENT INHIBITION OF LIPID PEROXIDATION MICROSOMES

Studies utilizing rat liver microsomes indicate that lipid peroxidation can be initiated by the addition of soluble fractions of rat liver homogenates. Kamataki et al. found that the addition of 1 mM GSH produced a 70 percent reduction in MDA produced by microsomal incubations over that of controls (23). Other sulfhydryl containing compounds such as cysteine, dithiothreitol and β-mercaptoethanol were much less effective in inhibiting NADPH induced microsomal lipid peroxidation. Protection against peroxidation also occurred following the addition of soluble fractions of rat liver homogenates. These researchers did not determine if the inhibitory nature of these soluble fractions was due to endogenous GSH, a cytosolic protein or a combination of these two factors. More definitive studies examining the role of each of these components were conducted by Gibson et al. (24). These researchers found that a heat labile, nondialyzable cytosolic factor inhibited lipid peroxidation in heated rat liver microsomes. The effectiveness of this factor was greatly increased if GSH or other small thiol groups were included in the reaction mixture. It was concluded from these studies that the factor is as a cytosolic protein with one or more sulfhydryl groups required for activity.

In addition to this cytosolic factor, there appears to be an microsomal protein which in the presence of GSH protects against lipid peroxidation in microsomes. Burk (25) showed that the addition of GSH to microsomal incubations inhibited both ascorbate and NADPH induced lipid peroxidation. In this system, GSH produced a lag period prior to the onset of lipid peroxidation. Once the lag period had ended, lipid peroxidation ensued at the same rate as in incubations containing no GSH. Increasing GSH concentrations produced longer lag periods prior to the onset of lipid peroxidation. The protective effects of GSH were abolished by exposure of microsomes to trypsin or heat and could not be duplicated by using other thiol containing compounds such as cysteine, dithiothreitol and propylthiouracil in place of GSH. Extensive washing of microsomes failed to remove the factor responsible for the GSH dependent protection.

Similar findings have been reported by Christophersen (26), Haenen and Bast (27) and Reddy et al. (28) with rat liver microsomes. Haenen and Bast (27) showed that the protective effects of GSH on microsomal lipid peroxidation could be abolished by exposure to heat or to lipid peroxidation prior to the addition of GSH. In vivo treatments of rats with carbon tetrachloride or phorone also diminished the protective effects of GSH on microsomal lipid peroxidation. These agents purportedly inhibited the factor by inducing lipid peroxidation in vivo. Reddy

et al. (28) determined that microsomes isolated from vitamin E deficient rats did not exhibit GSH dependent inhibition of lipid peroxidation. These researchers confirmed that the inhibition of lipid peroxidation was specific for GSH and could not be duplicated by other thiol containing compounds. The preceding reports suggest that GSH alone cannot inhibit lipid peroxidation. Rather, a protein acting in conjunction with GSH is required for the expression of GSH dependent protection. As discussed previously, both a microsomal and cytosolic protein have been implicated in this process. Differences in substrate specificity requirements tend to indicate that these two proteins are distinct. The microsomal protein, for example, exhibits an absolute requirement for GSH while the cytosolic protein can utilize a variety of thiol containing compounds of low molecular weight. The cytosolic protein protects against lipid peroxidation in heated microsomes. This treatment would be expected to inactivate the microsomal protein. Repeated washings also failed to remove the microsomal GSH dependent factor (24). All of these factors argue for the separate identity of these two proteins. At present, the relative contribution of the microsomal or cytosolic protein in inhibiting lipid peroxidation *in vivo* is unknown.

PROPOSED MECHANISMS OF GLUTATHIONE DEPENDENT INHIBITION OF PEROXIDATION

Regeneration of Vitamin E by Glutathione

Three distinct mechanisms have been proposed to explain the ability of GSH to inhibit lipid peroxidation. Reddy et al. (28) found no evidence of GSH dependent protection against lipid peroxidation in microsomes isolated from vitamin E deficient rats. On the basis of this observation, a mechanism whereby GSH regenerated vitamin E through the action of a GSH dependent microsomal protein was proposed. However, research has shown that the ability of GSH to protect against lipid peroxidation is also sensitive to lipid peroxidation (27). Rats fed a vitamin E deficient diet exhaled higher levels of ethane than those rats fed a diet supplemented with vitamin E (29). The vitamin E deficient rats utilized by Reddy et al. (28) may therefore have increased endogenous lipid peroxidation. This state may in turn inhibit the ability of the microsomal protein to protect against peroxidation.

The effects of vitamin E on the GSH protective microsomal protein were also examined by Hill and Burk (30). These researchers found that GSH inhibition of peroxidation still occurred in vitamin E deficient rats. Microsomes containing higher levels of vitamin E displayed progressively longer GSH induced lag periods before the onset of peroxidation. Digestions with trypsin destroyed

the GSH induced lag without altering vitamin E levels. Hill and Burk concluded that both vitamin E and GSH are effective in inhibiting microsomal lipid peroxidation but that they function independently.

Glutathione Peroxidase Dependent Inhibition of Lipid Peroxidation

Several reports have suggested a role for enzymes possessing glutathione peroxidase activity in the inhibition of lipid peroxidation. Glutathione peroxidase activity has been associated with both a selenium dependent and selenium independent group of proteins. Research has identified the selenium independent group of proteins as belonging to an assemblage of enzymes known as the glutathione transferases (31). Although collectively, the glutathione peroxidases comprise a diverse set of enzymes, a similar reaction mechanism has been proposed to account for their inhibition of peroxidation. This reaction involves the enzyme mediated reduction of lipid hydroperoxides to lipid alcohols with the concurrent oxidation of two molecules of GSH to oxidized glutathione (GSSG). Since lipid hydroperoxides have been shown to be involved in the propagation of lipid peroxidation (32), the reduction of lipid hydroperoxides would be expected to break the autocatalytic reactions involved in peroxidation and thereby inhibit lipid peroxidation. The classical

glutathione peroxidase is a soluble enzyme containing four atoms of selenium and exhibiting a molecular weight of about 75,000 (33).

Reports have indicated that lipid hydroperoxides in solution are substrates for both the classical glutathione peroxidase (34) and at least some of the soluble glutathione transferase isozymes (35). Despite this evidence, the ability of these enzymes to inhibit peroxidation remains controversial.

Wright et al. (36) examined the relative abilities of added GSH and cytosol to protect against NADPH induced peroxidation in hepatic microsomes. Although GSH inhibited peroxidation, rat liver cytosol containing similar quantities of GSH had little effect. Measurements detected significant quantities of glutathione peroxidase activity in the cytosolic fractions utilized in these experiments, but these enzyme levels apparently did not inhibit microsomal peroxidation. Beloqui and Cederbaum conducted similar experiments utilizing hepatic microsomes (37). The addition of GSH reduced the rate of NADPH catalyzed peroxidation by about 40 percent in these experiments. The addition of purified preparations of the soluble enzymes glutathione peroxidase, glutathione reductase and glutathione transferase together with GSH did not significantly increase the protective capacity of GSH in these incubations.

In contrast to these results, Burk (38) showed that

microsomal NADPH induced peroxidation could be inhibited, at least in part, by the addition of GSH along with partially purified glutathione transferase fractions. No inhibition of peroxidation was demonstrated in this system with fractions containing selenium dependent glutathione peroxidase activity.

Heat denatured hepatic microsomes initiated with ascorbate and ferric iron were not protected by the addition of glutathione transferase or glutathione peroxidase fractions in the presence of GSH (39). Significantly, little GSH peroxidase activity was detected when boiled peroxidized microsomal preparations were incubated with dialyzed cell sap and GSH. If however, microsomal phospholipids were solubilized prior to incubations with this system, considerable peroxidase activity was associated with the dialyzed cell sap. Thus, it was concluded that the inability of the selenium containing glutathione peroxidases and glutathione transferases to inhibit peroxidation may be related to the failure of these enzymes to interact with lipid hydroperoxides still associated with the lipophilic environment of biological membranes.

A series of experiments point to the importance of the enzyme phospholipase in the GSH dependent protection of microsomes against lipid peroxidation. Reports by Tan et al. (40) indicated that the addition of glutathione transferases B and AA inhibited NADPH induced lipid

peroxidation. If microsomal preparations were pretreated with the phospholipase A_2 inhibitor p-bromophenacyl bromide, no protection was observed with these enzymes. Glutathione transferase dependent protection was restored in this system, following p-bromophenacyl bromide treatment, by the addition of large quantities of porcine pancreatic phospholipase A_2. Glutathione transferases and glutathione peroxidases in the presence of GSH did not inhibit ascorbate induced peroxidation of heat inactivated microsomes. These heat inactivated microsomes had no phospholipase activity. Tan et al. (40) concluded that lipid peroxidation can be inhibited by the concerted actions of phospholipase A_2 and either the selenium dependent glutathione peroxidase or specific glutathione transferases. In this system, phospholipases are proposed to interact with lipid hydroperoxides and selectively act on these compounds to release them from the microsomal membrane. Once released, these hydroperoxides can become substrates for the soluble enzymes possessing glutathione peroxidase activity.

Although collectively, the studies described are somewhat contradictory, the preponderance of evidence suggests that the soluble selenium dependent glutathione peroxidase and glutathione transferases by themselves do not increase the capacity of GSH to inhibit microsomal lipid peroxidation. Central to this issue is the apparent inability of these soluble enzymes to interact with lipid

hydroperoxides in biological membranes. Although phospholipase A_2 may make these hydroperoxides more accessible to these enzymes, the role of the phospholipases in the GSH dependent inhibition of lipid peroxidation is not clear. As previously indicated, studies conducted by Wright et al. (36) and Beloqui and Cederbaum (37) detected no additional protection by GSH when glutathione peroxidase containing fractions were incubated with microsomes.

The microsomal preparations utilized in these experiments were not heat inactivated or pretreated with phospholipase inhibitors and should therefore possess active phospholipases. Thus, the inhibition of lipid peroxidation by enzymes possessing glutathione peroxidase activity may still not occur despite the actions of phospholipases.

In addition to the soluble glutathione peroxidases previously discussed, several studies suggest that GSH dependent peroxidase activity may also be associated with intracellular membranes. Ursini et al. (41) purified a selenium containing glutathione peroxidase from pig heart with a molecular weight of 23,000. Although the enzyme had a similar amino acid composition and contained selenium, differences in substrate specificities indicate that this enzyme is distinct from the classical glutathione peroxidase. Phospholipid hydroperoxides incorporated in liposomes could be reduced by this enzyme but not by the actions of the classical glutathione peroxi-

dase. Ursini et al. found that the activity of this enzyme was markedly stimulated toward a variety of hydroperoxides by the addition of the nonionic detergent Triton X-100. Based on this information, these researchers concluded that the peroxidase was interfacial in character. The addition of this membrane associated glutathione peroxidase as well as 5 mM GSH markedly inhibited the iron ascorbate induced peroxidation of phosphatidylcholine liposomes (42). Both oxygen consumption and MDA production were inhibited by the addition of GSH and the peroxidase in this experiment. Significantly, this inhibition did not require the addition of phospholipases.

This experiment provides strong evidence that a GSH dependent peroxidase can indeed inhibit lipid peroxidation. The interfacial character of the glutathione peroxidase may allow this enzyme to reduce phospholipid hydroperoxides still associated with the lipophilic environment of liposomes and thereby prevent peroxidation. However, the ability of this peroxidase to inhibit lipid peroxidation in vivo is unknown.

A membrane bound glutathione transferase exhibiting peroxidase activity has been purified from rat liver microsomes (43). The substrate specificity of this selenium independent enzyme is similar to those seen with the soluble glutathione transferases. For example, chlorodinitrobenzene and the nonpolar compound cumene

hydroperoxide have been demonstrated to be substrates for the microsomal transferase (44). This transferase is unique with respect to the soluble glutathione transferases in that it is activated by the sulfhydryl modifying reagent N-ethylmaleimide (45). At present, no research has investigated whether this protein can inhibit lipid peroxidation *in vivo* or *in vitro*.

Numerous reports indicate that GSH inhibits microsomal lipid peroxidation (25-28). This inhibition occurs without the addition of cytosolic enzymes and despite repeated washings to remove contaminating cytosolic enzymes (25). Although it is tempting to attribute this inhibition to a microsomal GSH dependent peroxidase activity, no evidence linking such a peroxidase activity with microsomal protection has been reported.

Glutathione Dependent Inhibition of the Initiation of Lipid Peroxidation

Gibson et al. (24,39) proposed that GSH protects microsomes against peroxidation by inhibiting the initiation of peroxidation. Studies conducted by this group indicate that dialyzed cell sap and GSH prevented the ascorbate induced lipid peroxidation of heat inactivated rat liver microsomes. This GSH dependent factor inhibited MDA production, oxygen consumption and conjugated diene production (24). In a similar set of experiments, the

addition of this cytosolic factor as well as GSH protected microsomes against NADPH induced peroxidation (46). Analysis of fatty acids by thin layer chromatography failed to detect measurable quantities of hydroxy fatty acids in microsomes protected by GSH (46). Based on these studies, it was concluded that the GSH dependent cytosolic factor must inhibit microsomal lipid peroxidation by inhibiting the initiation of peroxidation (24,36,46) these researchers hypothesized that if the cytosolic factor acted by reducing lipid hydroperoxides significant oxygen consumption and conjugated diene production should occur (24). It was also concluded that the product of a peroxidase reaction, hydroxy fatty acids, should also be present in microsomal extracts if the cytsolic factor is indeed a glutathione peroxidase (46).

Research has shown that lipid hydroperoxides are involved in the propagation of lipid peroxidation (32). If these lipid hydroperoxides are efficiently reduced, the propagation phase of lipid peroxidation may be checked. The levels of fatty alcohols present in lipids may depend to a large degree on what stage of lipid peroxidation the peroxidase functions. If lipid hydroperoxides are reduced efficiently during the early stages of propagation, little fatty alcohols and conjugated dienes may be produced. These products may in fact only be detected during the latter stages of lipid peroxidation where large amounts of lipid hydroperoxides are formed. Experiments by Ursini et

al. (42) provide support for this hypothesis. These researchers demonstrated that the addition of GSH and a GSH dependent peroxidase inhibited the peroxidation of phosphatidylcholine liposomes. Significantly, no fatty alcohols were detected in these incubations despite the addition of the peroxidase. Therefore, the failure to detect fatty alcohols during the GSH dependent inhibition of peroxidation does not necessarily eliminate the involvement of a GSH dependent peroxidase activity in the inhibition of peroxidation.

THE IMPORTANCE OF NUCLEAR LIPID PEROXIDATION

Lipid peroxidation has been demonstrated to occur in such subcellular organelles as lysosomes, mitochondria, and microsomes (1). Few studies, however, have focused on the susceptibility of the cell nucleus to lipid peroxidation.

The nucleus is surrounded by a complex double membrane system known as the nuclear membrane or envelope. Within the cell, the outer nuclear membrane is closely associated with the membrane of the endoplasmic reticulum (47). At certain sites on the nuclear envelope, the outer and inner nuclear membrane are joined to form nuclear pores. The fusion of the outer and inner nuclear membranes also produces a perinuclear cisternae. The luminal width of the intracisternal space varies according to the cell type examines, but typically it ranges from 100 to 600 angstroms (47).

The nuclear membrane system has been implicated in a variety of critical cellular functions (47). Reports have suggested that mRNA is transported from the nucleus to the cytoplasm through nuclear pore complexes. The nuclear membrane system may also contribute to the organization of chromatin and may be involved in nuclear division. It seems likely that nuclear lipid peroxidation may be disruptive to many of these critical functions.

Lipid peroxidation produces numerous reactive

chemical species. Lipid hydroperoxides, lipid radicals (48), and such activated oxygen species as superoxide anion, singlet oxygen (49) and hydroxyl radicals (50) may all be products of lipid peroxidation. In addition to these products, the decomposition of lipid hydroperoxides can yield carbonyl compounds such as MDA and hydroxyalkenals (51).

In recent years, numerous studies have indicated that lipid peroxidation or lipid peroxidation products can alter the structure and function of DNA. Inouye (52) demonstrated that the lipid hydroperoxide 13-L-hydro-peroxy-cis-9, trans-11-octadecadienoic acid produced site specific breaks in double stranded DNA. Similarly, DNA fluoresced after exposure to lipid hydroperoxides. This fluorescence increased significantly if ascorbate and iron were added to the incubation mixture (53). Studies conducted by Ueda et al. (54) indicate that autoxidized lipids incubated with DNA and iron caused DNA strand breaks. The addition of the hydroxyl radical scavenger, potassium iodide, or the superoxide scavenger, Tiron, to the incubation mixture almost completely prevented DNA strand scission. On the basis of these data, Ueda et al. concluded that oxygen radicals or other radical species were involved in the cleavage of DNA.

Further support for the involvement of oxygen radicals in DNA strand scission was provided by Brawn and Fridovich (55). Incubation of DNA with xanthine and the

enzyme xanthine oxidase produced DNA strand breaks. DNA was protected by the addition of superoxide dismutase, catalase or hydroxyl radical scavengers. Superoxide or hydrogen peroxide induced single strand breaks in purified phage DNA in research conducted by Lesko et al. (56). This superoxide or hydrogen peroxide induced DNA damage did not occur if hydroxyl radical scavengers were included in the incubation mixture. Both of these studies suggest that hydroxyl radicals may be ultimately responsible for the cleavage of DNA by activated oxygen species.

The lipid peroxidation decomposition products MDA and hydroxyalkenals have also been shown to alter DNA. Malondialdehyde was found to be mutagenic to the bacterium Salmonella tyhimurium (57). Analogous results were seen with the carbonyl compounds hydroxyalkenals. Brambilla et al. (51) determined that certain hydroxyalkenals fragmented DNA and produced sister-chromatid exchange in Chinese hamster ovary cells.

Considerable evidence suggests that lipid peroxidation or lipid peroxidation products can alter the integrity of DNA in vitro. Still, little is known concerning the potential of lipid peroxidation to damage DNA in vivo. Numerous studies indicate that antioxidants can inhibit chemically induced carcinogenesis (58). This inhibition has been demonstrated with a wide variety of xenobiotic carcinogens. The inhibition of carcinogenesis by antioxidants may point to the importance of chemically

induced lipid peroxidation in mediating carcinogenesis. Furthermore, since DNA damage has been strongly linked with the onset of carcinogenesis, the interaction of lipid peroxidation products with DNA may be important in the induction of carcinogenesis. Although this explanation is an attractive hypothesis, it remains highly speculative. Further experimentation is required to assess the ability of lipid peroxidation to damage DNA in vivo and contribute to the process of carcinogenesis.

Lipid peroxidation occurring throughout the cell may produce reactive compounds which subsequently diffuse into the nucleus and damage nuclear constituents. The proximity of the nuclear membrane may be especially important in contributing to the reaction of lipid peroxidation products with DNA or DNA utilizing enzymes. Several reports, discussed previously, have implicated activated oxygen species such as hydroxyl radical as being the agents responsible for DNA damage. Hydroxyl radicals are extremely reactive agents. Studies have estimated that the hydroxyl radical may diffuse only about 60 angstroms before reacting with cellular constituents (59). Segments of DNA are closely associated with the inner nuclear membrane (47) and may therefore be at an increased risk from nuclear peroxidation.

The proximity of the nuclear membrane to such critical cellular targets as DNA may also contribute to the interaction of more stable lipid peroxidation products

with DNA. Many of these stable lipid peroxidation products are substrates for the cytosolic enzymes aldehyde dehydrogenase (60), glutathione transferase (61) and the selenium dependent glutathione peroxidase (34). Lipid peroxidation products generated throughout the cell must diffuse through the cytosol before entering the nucleus. In so doing, these compounds may become substrates for these protective cytosolic enzymes. Lipid peroxidation products generated in the nuclear membrane would be required to diffuse only short distances before interacting with DNA and may thereby escape the action of these cytosolic enzymes.

The following dissertation examines the ability of GSH to protect against nuclear membrane peroxidation and the mechanism involved the this protection.

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CHARACTERIZATION OF GLUTATHIONE DEPENDENT INHIBITION OF LIPID PEROXIDATION OF ISOLATED RAT LIVER NUCLEI

ABSTRACT

Glutathione (GSH) is known to play an important role in protecting cells against oxidative stress. The present study was undertaken to assess the ability of GSH to protect isolated rat liver nuclei against lipid peroxidation. Nuclei were isolated from rat liver homogenates by discontinuous sucrose gradient centrifugation, and lipid peroxidation was induced by 1.7 mM ADP, 0.11 mM EDTA, 0.1 mM $FeCl_3$, and either 1 mM NADPH or 0.5 mM ascorbate. The amount of lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive products and the disappearance of lipid unsaturated fatty acid moieties. The addition of GSH (0.1 to 1.0 mM) produced a concentration dependent lag period prior to the onset of lipid peroxidation. This GSH induced lag period was abolished by pretreatment of nuclei with trypsin, thiol modifying reagents, disulfides, or heating nuclei at 60°C for 15 min. Nuclei which were incubated with GSH also catalyzed the conversion of cumene hydroperoxide to cumyl alcohol. Similarly, this activity was also inhibited by thiol modifying reagents, disulfides, and heating nuclei at 60°C for 15 min. The data suggest that a GSH dependent peroxidase activity is

associated with rat liver nuclear membranes which is capable of inhibiting lipid peroxidation.

INTRODUCTION

Lipid peroxidation is thought to be an important biological consequence of oxidative cellular damage (1). The destruction of unsaturated fatty acids, which occurs in lipid peroxidation, has been linked with altered membrane structure (2) and enzyme inactivation (3). In addition to lipid hydroperoxides and lipid radicals (4), lipid peroxidation may generate activated oxygen species such as hydroxyl radicals (5) and superoxide anions (6). The decomposition of peroxidized polyunsaturated fatty acids also generates reactive carbonyl compounds such as malondialdehyde (MDA) and hydroxyalkenals (7). Several studies indicate that such lipid peroxidation products can alter the structure and function of DNA (8-11).

Evidence strongly suggests that lipid peroxidation can cause extensive damage to subcellular organelles and biomembranes. Lipid peroxidation has been demonstrated to occur in isolated mitochondria, lysosomes and microsomes (12). Few studies, however, have focused on the susceptibility of the cell nucleus to lipid peroxidation. The nuclear membrane regulates the transport of mRNA into the cytoplasm and aids in the process of nuclear division. DNA is also frequently associated with certain regions of the nuclear membrane (13). It seems likely that nuclear membrane peroxidation may disrupt many of these critical functions. The proximity of the nuclear membrane to DNA

could also contribute to the interaction of DNA with reactive compounds generated in lipid peroxidation. This fact is of importance since research indicates that hydroxyl radicals diffuse an average of only 60 angstroms before reacting with cellular components (14). Nuclear peroxidation may also increase interactions between more stable peroxidation products and DNA. The cytosolic enzymes aldehyde dehydrogenase (15), glutathione transferase (16) and glutathione peroxidase (17) have all been shown to metabolize various reactive lipid peroxidation products. Such cytosolic enzymes may metabolize peroxidation products generated throughout the cell before they diffuse into the nucleus and interact with DNA. The proximity of the nuclear membrane to DNA may limit the ability of these cytosolic enzymes to metabolize products generated by nuclear lipid peroxidation.

Several reports suggest that glutathione (GSH) either alone (18) or in conjunction with added proteins (19, 20) can protect microsomes against lipid peroxidation. A report by Burk (18) indicates that GSH inhibits microsomal lipid peroxidation. This protection did not require the addition of other proteins. However, his evidence did suggest the involvement of a microsomal protein. Ursini et al. (21) recently isolated from pig heart a protein which displays glutathione peroxidase activity toward cumene hydroperoxide, hydrogen peroxide and lipid hydroperoxides and is distinct from the classical glutathione

peroxidase (22). Evidence suggests that the enzyme is interfacial in character and can interact directly with liposomes to reduce phospholipid hydroperoxides (21). The addition of this protein to microsomal incubation mixtures inhibited lipid peroxidation (19). Gibson et al. (20) have reported that a cytosolic, GSH dependent protein can protect microsomal membranes against peroxidation. These researchers, however, concluded that this protection was not associated with glutathione peroxidase activity but rather involved the inhibition of the initiation of peroxidation.

The following study was conducted to determine if GSH can protect isolated nuclei against lipid peroxidation and whether this protection involves the action of a GSH dependent peroxidase activity. The loss of unsaturated fatty acids and MDA production were both utilized to monitor NADPH induced peroxidation and to examine the protective effects of GSH. Results from this study suggest that nuclei are susceptible to NADPH induced lipid peroxidation and that GSH inhibits this peroxidation through a GSH dependent peroxidase activity.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats (Simonsen Labs., Gilroy, CA) (250-300 g) were used throughout the course of this study. These animals had free access to Purina Rat Chow and water.

Chemicals

NADPH (type I), trypsin (type III-s), and trypsin inhibitor (type II-s) were purchased from Sigma Chemical Company (St. Louis, MO). Standard fatty acid methyl esters and diheptadecanoin were obtained from Nu Chek Prep Inc. (Elysian, MN). The silylation reagent mixture N,O,-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Pierce Chemical Company (Milwaukee, WI). Desferal was a generous gift from the CIBA Pharmaceutical Company (Summit, NJ). All other chemicals were obtained from Sigma Chemical Company.

Preparation of Nuclei

Animals were anesthetized with ether then decapitated. Livers were immediately removed and placed on ice. Nuclei were isolated from rat liver according to Blobel and Potter (23) with slight modifications for large scale preparations utilizing a Beckman SW 28 rotor. After isolation, nuclei were resuspended in 30 ml 100 mM NaCl

buffer TM[1] and centrifuged at 4000 x g for 10 min at 4°C. Nuclei were washed twice in this manner prior to all incubations to insure preparations were free of cytosolic proteins.

Incubation Conditions

All incubations were conducted in 100 mM NaCl buffer TM at 37°C. The NADPH induced peroxidation system consisted of 1 mM NADPH, 1.7 mM ADP, 0.11 mM EDTA and 0.1 mM $FeCl_3$ (24). The ascorbate induced peroxidation system consisted of the same components but was initiated with 0.5 mM ascorbate instead of 1 mM NADPH. Peroxidation was initiated at time zero by the addition of 1.7 mM ADP, 0.11 mM EDTA and 0.1 mM $FeCl_3$. Zero time points were taken immediately after the addition of the ADP, EDTA and $FeCl_3$ solution.

Analysis of Nuclear Fatty Acid Composition

Nuclear lipids were extracted according to the procedures of Folch et al. (25). Using the method developed by Metcalfe et al. (26), extracted nuclear lipids were then saponified and derivatized to fatty acid methyl esters. Diheptadecanoin was added prior to derivatization and subsequently used as an internal standard. Following derivatization, samples were evaporated under N_2 and resuspended in appropriate volumes of iso-octane.

Fatty acid methyl esters were analyzed using a gas

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[1]50 mM Tris, 5 mM $MgCl_2$ pH 7.5

chromatograph equipped with a flame ionization detector. Samples were injected on a 30 m fused silica capillary column (Supelcowax 10, Supelco Inc.) with an inner diameter of 0.25 mm. The column was initially maintained at 180°C for 3 min followed by an increase in temperature at a rate of 1°C/min until a final temperature of 220°C was achieved.

Extraction of Nuclear Suspensions for the Determination of Cumene Hydroperoxide Levels

At designated times, aliquots of nuclear suspensions were added to tubes containing 5 ml hexane. The tubes were then immediately mixed for 10 sec, sonicated for 40 sec, and finally mixed for an additional 50 sec. Following brief centrifugation, the hexane layer was transferred to tubes containing 0.35 ml methanol. The hexane-methanol mixture was then evaporated under N_2 to a volume of 0.35 ml. When aqueous solutions contained Triton X-100, chloroform was utilized instead of hexane in the above extraction procedures to prevent micelle formation.

Derivatization of Cumyl Alcohol

Following incubations, aliquots of nuclear suspensions were extracted with hexane according to the procedures developed for cumene hydroperoxide. However, 0.5 ml pyridine was added to the hexane layer instead of 0.35 ml methanol to facilitate subsequent silylation of the cumyl alcohol. The hexane-pyridine mixture was evaporated to a volume of 0.5 ml. This volume was then

mixed with 0.5 ml BSTFA containing 1% TMCS and heated at 70°C for 15 min to prepare trimethyl silylated derivatives. Standard trimethyl silylated derivatives of cumyl alcohol were prepared in a similar manner utilizing the compound 2-phenyl-2-propanol.

Samples were injected on a gas chromatograph equipped with a DB5 (J and W Company) 30 m fused silica capillary column. A temperature gradient of 50-200°C at 10°C/min was utilized. Detection was achieved with a Finnigan (model 4023) mass spectrometer with a 4500 model source operated at 150°C. An electron impact energy of 50 eV was used.

Chemical Analysis

Proteins were determined by the method of Lowry et al. (27) as described by Peterson (28). Phosphorus levels were assayed using the procedures of Fiske and Subbarow (29) as modified by Bartlett (30). Both GSH and glutathione disulfide (GSSG) levels were measured by HPLC analysis with UV detection (31) as modified by (32). Peroxide levels were spectrophotometrically determined according to the procedures of Hicks and Gebicki (33).

Malondialdehyde levels were determined by using the thiobarbituric acid assay as developed by Wills (34) with slight modifications. Aliquots (0.25 ml) of nuclear suspensions were added to 0.5 ml 10% (w/v) trichloracetic acid. The resulting mixture was chilled on ice then centrifuged for 2 min at 15,000 x g. Following centri-

fugation, supernatants were removed and added to 1.0 ml of 0.67% (w/v) thiobarbituric acid. Samples were heated at 95°C for 20 min and then cooled to room temperature. Absorbance values were measured at 532 nm.

RESULTS

Effects of GSH on the NADPH Induced Loss of Polyunsaturated Fatty Acids

Lipid extracts from isolated nuclei were analyzed for their fatty acid composition. As shown in Table II-1, prior to incubations, nuclei contained 45.8% (weight percent) PUFA. Incubation of isolated nuclei with the NADPH peroxidation system resulted in time dependent decreases in the level of PUFA. For example, after 60 min, only 24.6% of the total fatty acids analyzed were polyunsaturated. Addition of GSH to the incubations inhibited lipid peroxidation and limited the decrease in PUFA to 37.7% of the total after 60 min. In this study, peroxidation of fatty acids occurred primarily in the arachidonic and docosahexaenoic acid fractions. Little loss of the unsaturated oleic and linoleic acid fractions was observed regardless of the presence of GSH. Control nuclei, containing all of the components of the peroxidation system except NADPH, exhibited little loss of PUFA even after 60 min of incubation.

Effects of GSH and Other Thiol Containing Compounds on MDA Production in Isolated Nuclei

Malondialdehyde levels were monitored in incubations containing GSH, nuclear suspensions, and the NADPH peroxidation system (Figure II-1). Incubation conditions were the same as in Table II-1 except MDA formation was uti-

TABLE II-1

Effects of NADPH Induced Lipid Peroxidation and of GSH on the Fatty Acid Composition of Nuclear Lipids[a]

Fatty Acid[c]	No Incubation (%)[d]	Incubation[b] 30 min (%) -GSH	30 min (%) +GSH	60 min (%) Control[e]	60 min (%) -GSH	60 min (%) +GSH
16:0	23.4	28.6	25.6	22.1	35.9	27.3
18:0	22.8	25.3	25.9	25.9	26.9	27.3
18:1	8.1	11.5	7.6	6.7	11.7	8.4
18:2	17.5	16.7	14.3	16.5	16.3	15.8
20:4	22.8	14.7	21.2	21.9	8.3	17.4
22:6	5.5	3.3	5.7	6.8	N.D.[f]	4.1
% Polyunsaturated Fatty Acid:	45.8	34.7	41.2	45.2	24.6	37.3

a Nuclear lipids were extracted and derivatized to fatty acid methyl esters as described in the experimental procedures section.

b Incubations were conducted with 1 mM GSH (+GSH) or without GSH (-GSH) as indicated and the NADPH peroxidation system. The incubation buffer and the NADPH peroxidation system are described in the experimental procedures section

c Number of carbon atoms:Number of double bonds.

d (%)-Weight percent.

e Control incubations contained all the components of the NADPH peroxidation system except NADPH. No GSH was added to these incubations.

f Not detectable.

Figure II-1. The effects of GSH on NADPH induced nuclear lipid peroxidation. Nuclear suspensions were incubated with the NADPH peroxidation system. Both nmol MDA/mg protein (——) and % GSH remaining in the reduced form (– – –) are plotted verses time of incubation. (◇)- no GSH, (□,■)- 0.1 mM GSH, (△,▲)- 0.5 mM GSH and (○,●)- 1.0 mM GSH. Filled symbols represent data points for the % GSH remaining in the reduced form verses time plot. The incubation conditions and the NADPH peroxidation system are described in the experimental procedures section.

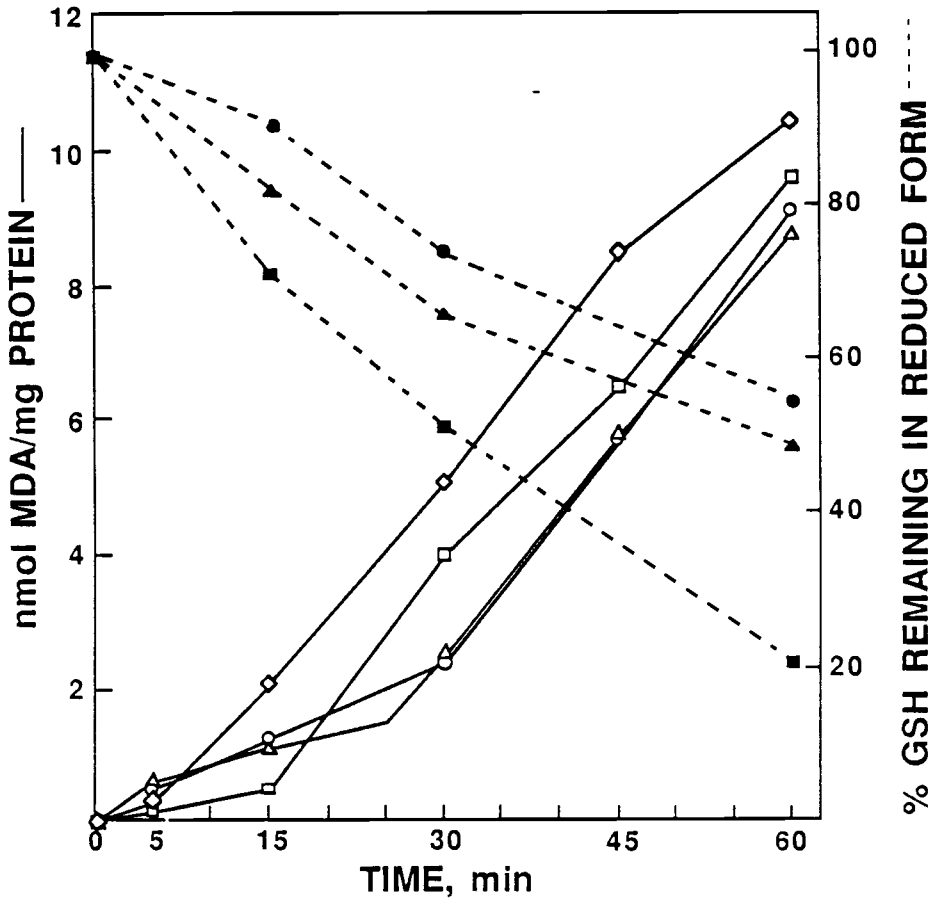
12
10
8
6
4
2
nmol MDA/mg PROTEIN
100
80
60
40
20
% GSH REMAINING IN REDUCED FORM
0
5
15
30
45
60
TIME, min

Figure II-1

lized to measure peroxidation. In control incubations, containing the NADPH system but no GSH, MDA levels increased after an initial lag of 5 min. The addition of GSH lengthened this initial lag and delayed the onset of rapid MDA formation. Once the initial GSH induced lag had ceased, MDA was produced at the same rate as in control incubations. Some MDA formation occurred in the presence of GSH but at much lower rates than present in control nuclei. Only low levels of MDA were detected in suspensions of isolated nuclei containing all of the components of the peroxidation system except NADPH (data not shown).

The concentration of GSH added to the incubation mixtures influenced the duration of the GSH induced lag period. Higher concentrations of GSH produced longer lag periods before the onset of lipid peroxidation. However, the duration of the GSH induced lag periods were not strictly proportional to initial concentrations of GSH. While 0.5 mM GSH caused a 25 min lag, doubling the concentration of GSH to 1 mM extended the lag to only 30 min. Even concentrations of GSH as low as 0.1 mM caused a 15 min lag prior to the rapid increases in MDA levels.

The rate of oxidation of GSH to GSSG was analyzed throughout the course of these experiments. The percent glutathione remaining in the reduced form (GSH) is plotted versus time in Figure II-1. The rate of GSH oxidation to GSSG was dependent on the initial concentration of GSH

present in the incubation mixture. Higher initial concentrations of GSH yielded lower rates of oxidation of GSH to GSSG.

Several other thiol-containing compounds were tested to determine their ability to inhibit lipid peroxidation (Table II-2). At the concentrations examined, cysteine, dithiothreitol (DTT) and β-mercaptoethanol did not delay the onset of NADPH induced lipid peroxidation. Therefore, inhibition of lipid peroxidation in the nuclear peroxidation system was specific for GSH.

Effects of Protein Modification Treatment on the Ability of GSH to Inhibit Peroxidation

A series of experiments were conducted to determine if the GSH dependent inhibition of peroxidation was enzyme mediated. Microsomal NADPH induced peroxidation is known to require the action of the enzyme cytochrome P-450 reductase (35). Any treatment designed to alter the integrity of proteins may also inhibit nuclear NADPH induced peroxidation. Therefore, a non-enzymatic system containing 0.5 mM ascorbate instead of 1 mM NADPH was used to initiate lipid peroxidation in these experiments. Isolated nuclei incubated with the ascorbate peroxidation system also exhibited GSH dependent inhibition of lipid peroxidation. GSH at concentrations of 1 mM produced a lag of 30 min prior to the onset of peroxidation (Figure II-2, 0 time). Heating isolated nuclei at 60°C produced a time dependent loss of this lag in peroxidation (Figure

TABLE II-2

Effects of Thiol Containing Compounds on NADPH Induced Peroxidation*

Thiol	Initial Conc. (mM)	Duration of Lag Period Prior to Onset of Lipid Peroxidation (min)
GSH	1	30
DTT	1	5
β-mercaptoethanol	1	5
Cysteine	2	5
No addition	-	5

* Incubation conditions and the NADPH peroxidation system are described in the experimental procedures section.

Figure II-2. The effects of heat (60°C) on GSH dependent protection against ascorbate induced peroxidation. Nuclei suspended in incubation buffer were heated at 60°C for (□)- 0 min, (■)- 1 min, (▲)- 4 min or (●)- 15 min. Following heating, the suspensions were cooled to 37°C then incubated with 1 mM GSH and the ascorbate peroxidation system. The incubation conditions and the ascorbate peroxidation system are described in the experimental procedures section.

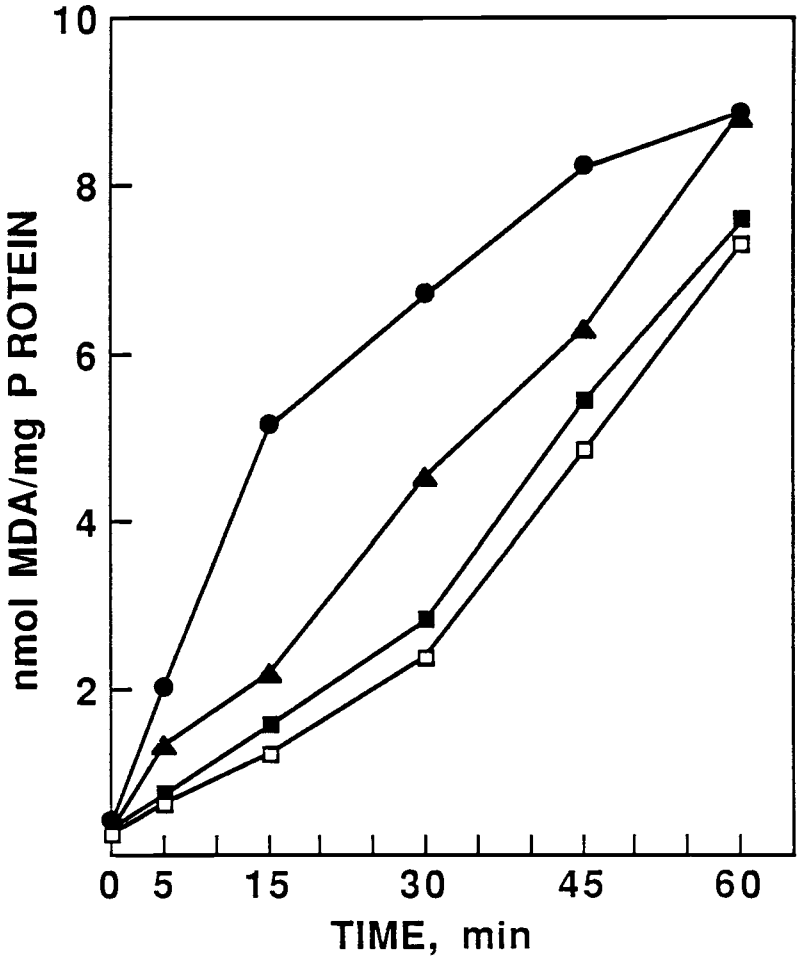

Figure II-2

II-2), such that heating nuclei for 15 min completely destroyed the protective effects of GSH on the ascorbate peroxidation system.

Pretreatment of nuclei with trypsin also yielded time dependent decreases in GSH protection (Figure II-3). Exposure of isolated nuclei to trypsin for as little as 1 min shortened the GSH induced lag in peroxidation, and exposure to trypsin for 5 min totally abolished the GSH effect.

Effect of Disulfides and Thiol Modifying Reagents on GSH Induced Inhibition of Peroxidation

Pretreatment of nuclei with 0.5 mM cystine abolished the GSH effect upon subsequent incubations with the NADPH peroxidation system (Table II-3). When lower concentrations of cystine were added during preincubations, longer GSH induced lag periods occurred.

The inhibition of the protective effect of GSH by cystine was reversible. In this experiment, isolated nuclei were preincubated with 0.5 mM cystine for 15 min at 37°C. Following the incubation, nuclei were pelleted and then incubated with 10 mM DTT for 15 min at 37°C. Nuclei were pelleted once again following the second preincubation and exposed to the NADPH peroxidation system and 1 mM GSH. The GSH induced lag period was restored to the full 30 min following these procedures (data not shown). Even at concentrations of 10 mM, DTT was unable to inhibit NADPH induced peroxidation without the addition of GSH.

Figure II-3. The effects of trypsin on GSH dependent protection against ascorbate induced peroxidation. Nuclei (3 mg protein/ml) were suspended in incubation buffer containing trypsin (0.3 mg/ml) and swirled at 37°C. After a (□)- 0 min, (■)- 1 min, (▲)- 5 min or (●)- 20 min digestion with trypsin, nuclei were mixed with trypsin inhibitor (0.6 mg/ml) then chilled on ice. Nuclear suspensions were centrifuged at 15,000 x g for 2 min. Supernatants were discarded, and pellets were resuspended in incubation buffer containing 1 mM GSH and the ascorbate peroxidation system. The incubation conditions and the ascorbate peroxidation system are described in the experimental procedures section.

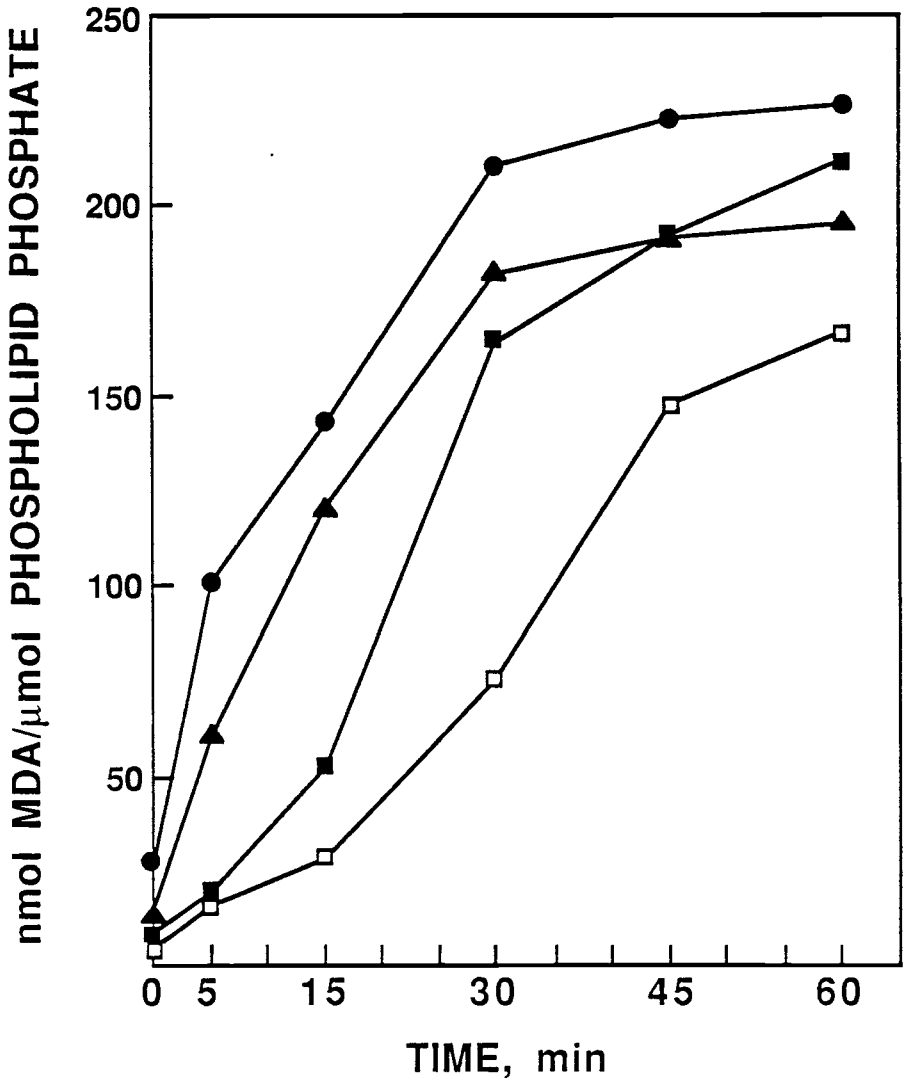
nmol MDA/μmol PHOSPHOLIPID PHOSPHATE
250
200
150
100
50
0
5
15
30
45
60
TIME, min

Figure II-3

TABLE II-3

Effects of disulfides and thiol modifying reagents on GSH dependent inhibition of lipid peroxidation

Preincubation Conditions[a]	Concentrations (mM)	Duration of Lag Prior to the Onset of Peroxidation (min)
Disulfides:[c]		
Cystine	0.5	0
Cystine	0.1	10
Cystine	0.05	20
GSSG	10.0	0
GSSG	5.0	5
GSSG	1.0	15
GSSG	0.25	23
Cystamine	5.0	0
Cystamine	1.0	15
Cystamine	0.5	25
No Addition	---	30
Thiol Modifying Reagents:[d]		
NEM	0.6	0
No Addition	---	25

[a] Preincubation conditions consisted of nuclei suspended in incubation buffer containing the disulfide or thiol modifying reagent and incubated at 37°C for 15 min.

[b] Following preincubation, nuclei were pelleted and then resuspended in incubation buffer containing 1 mM GSH and either the NADPH or ascorbate peroxidation system as indicated.

[c] NADPH peroxidation system.

[d] Ascorbate peroxidation system.

Therefore, DTT reverses the effects of cystine without otherwise affecting peroxidation.

Other disulfides showed similar effects as those displayed by cystine. Both GSSG and cystamine reduced the GSH dependent inhibition of lipid peroxidation. The major difference between these three disulfides involved the concentrations required to produce inhibition of the GSH effect. The concentrations of the disulfides required to prevent the GSH induced lag were 0.5 mM cystine, 10 mM GSSG and 5 mM Cystamine.

Preincubation of nuclear suspensions with the thiol modifying reagent, N-ethylmaleimide (NEM), inhibited NADPH induced peroxidation (data not shown). Therefore, the ascorbate peroxidation system was utilized in conjunction with NEM pretreatment. As can be seen in Table II-3, pretreatment of nuclei with 0.6 mM NEM eliminated GSH dependent inhibition of peroxidation.

As hydroperoxides were formed during lipid peroxidation and also serve as substrates for glutathione peroxidase, the effects of cumene hydroperoxide on the GSH dependent inhibition of nuclear peroxidation were assessed. Preincubation of nuclear suspensions with cumene hydroperoxide reduced the protective effects of GSH when nuclei were exposed to the NADPH dependent peroxidation system (Figure II-4). Concentrations of cumene hydroperoxide of only 0.5 mM abolished the GSH induced lags typically found in such incubations. Still higher

Figure II-4. The effects of cumene hydroperoxide preincubation on GSH dependent protection against NADPH induced peroxidation. Nuclei suspended in incubation buffer containing 50 uM desferal were preincubated with (□)- no cumene hydroperoxide, (■)- 0.025 mM cumene hydroperoxide, (▲)- 0.05 mM cumene hydroperoxide or (●) - 0.25 mM cumene hydroperoxide for 15 min. Following preincubation, nuclear suspensions were centrifuged at 4000 x g for 10 min. After centrifugation, supernatants were discarded and nuclear pellets were resuspended in the incubation buffer containing 1 mM GSH and the NADPH peroxidation system. The incubation conditions and the NADPH peroxidation system are described in the experimental procedures section.

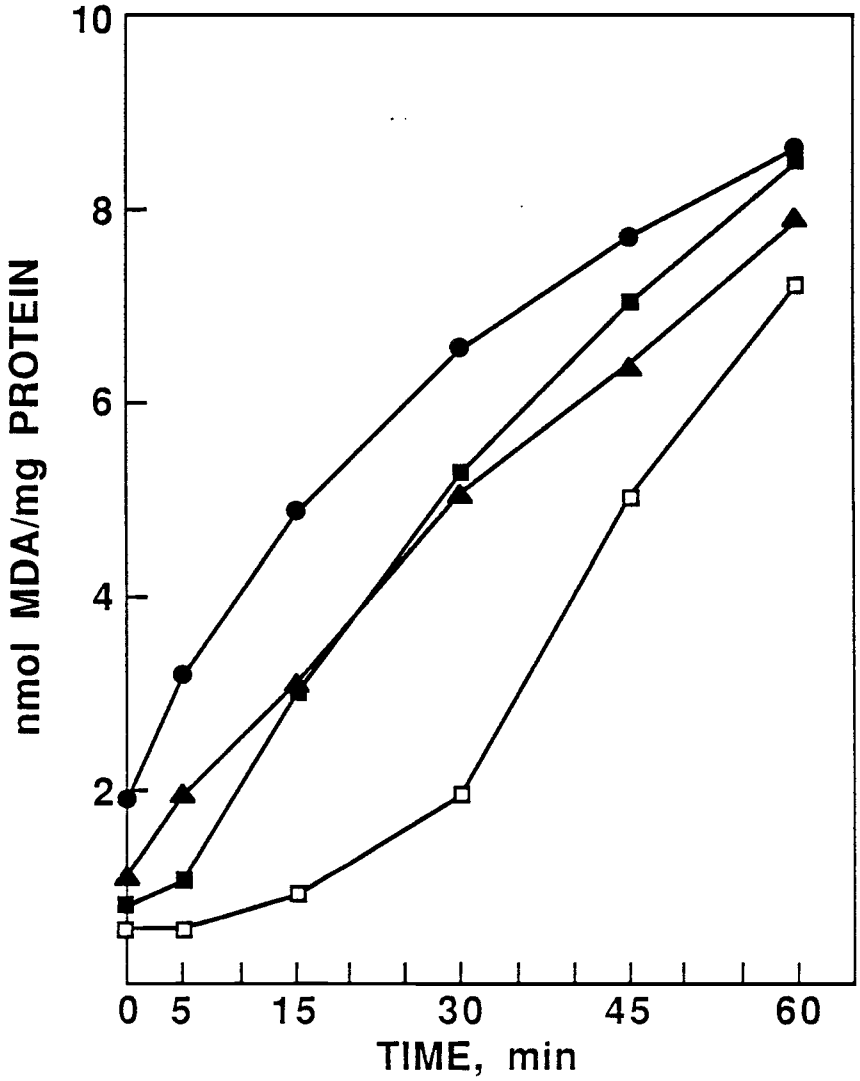

Figure II-4

concentrations of cumene hydroperoxide induced alterations in rates of MDA formation and increased levels of MDA detected at zero time.

The addition of GSH to the preincubation buffer along with cumene hydroperoxide greatly reduced the inhibitory effects of cumene hydroperoxide (Figure II-5). Under these preincubation conditions, concentrations of cumene hydroperoxide of 0.5 mM were now required to abolish the protective effects of GSH against peroxidation. Preincubations with GSH and cumene hydroperoxide also reduced initial MDA levels and restored normal rates of MDA formation.

Decreases in Cumene Hydroperoxide Levels During Incubations with Isolated Nuclei and GSH

Cumene hydroperoxide levels decreased in a time dependent manner in nuclear suspensions containing GSH (Table II-4). Indeed after 15 min, the hydroperoxide could no longer be detected. In incubations without GSH, little loss of the cumene hydroperoxide occurred even in the presence of nuclei. Decreases did occur in hydroperoxide levels when only GSH and no nuclei were present in the incubation mixture. Under these conditions, quantities of cumene hydroperoxide were reduced to about 43% of initial values following the end of 15 min. Thus, GSH reacted non-enzymatically with cumene hydroperoxide, and nuclei catalyzed this reaction.

Nuclei incubated with 1 mM GSH and 0.3 mM cumene

Figure II-5.The effects of GSH and cumene hydroperoxide preincubation on GSH dependent protection against NADPH induced peroxidation. Nuclei suspended in the incubation buffer containing 1 mM GSH and 50 uM desferal were preincubated with (□)- no cumene hydroperoxide, (■)- 0.1 mM cumene hydroperoxide, (▲)- 0.25 mM cumene hydroperoxide or (●)- 0.5 mM cumene hydroperoxide for 15 min. Following preincubation, nuclear suspensions were centrifuged at 4000 x g for 10 min. After centrifugation, supernatants were discarded and nuclear pellets were resuspended in the incubation buffer containing 1 mM GSH and the NADPH peroxidation system. The incubation conditions and the NADPH peroxidation system are described in the experimental procedures section.

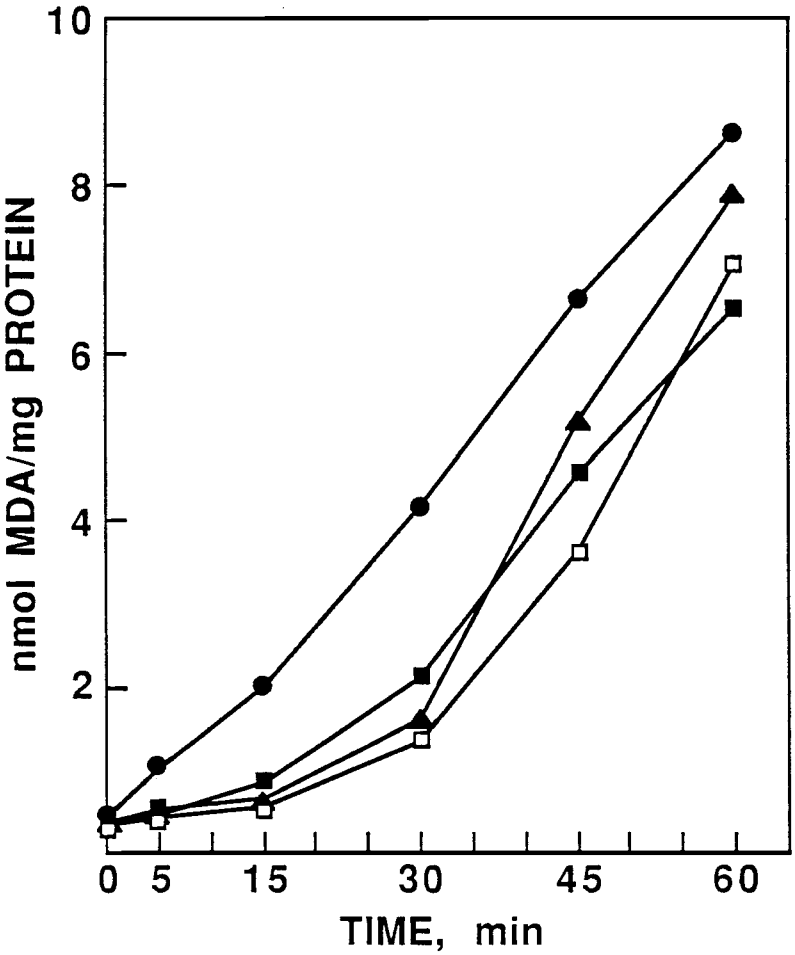

Figure II-5

TABLE II-4

Time Dependent Decreases in Cumene Hydroperoxide Levels During Incubation at 37°C[a]

Time of Incubation (sec)	+GSH		-GSH	
	+Nuclei	-Nuclei[b]	+Nuclei	-Nuclei[b]
	Cumene Hydroperoxide (umol)		Cumene Hydroperoxide (umol)	
10	0.48	1.03	1.25	1.10
60	0.33	1.12	1.15	1.03
180	0.12	0.92	1.11	1.14
900	N.D.[c]	0.52	1.07	1.16

[a] Nuclei were resuspended in incubation buffer containing 1 mMGSH (+GSH) or without (-GSH) and incubated at 37°C. 1.20 umol cumene hydroperoxide were added at time zero.

[b] -Nuclei indicates no nuclei were added to the incubation buffer.

[c] Not detectable (less than 0.02 umol).

hydroperoxide for 15 min were subsequently extracted, silylated and analyzed by gas chromatography with mass spectral detection. Large quantities of cumyl alcohol were detected in nuclear suspensions containing GSH and cumene hydroperoxide. Much smaller quantities of cumyl alcohol were found in incubation mixtures containing nuclei, cumene hydroperoxide but no GSH (data not shown).

As previously reported, a variety of treatments inhibited the GSH dependent inhibition of peroxidation. The effects of such treatments were tested on the ability of nuclear suspensions to reduce cumene hydroperoxide levels (Table II-5). Prior incubation of nuclei with 0.5 mM cystine, 0.6 mM NEM, and heating at 60°C for 15 min abolished the protective effects of GSH against lipid peroxidation. As Table II-5 indicates, these pretreatments also inhibited the ability of nuclei to catalyze the loss of cumene hydroperoxide in the presence of GSH.

Extraction of nuclei with incubation buffer containing 0.3% Triton X-100 solubilized the peroxidase activity which was active towards cumene hydroperoxide. Following such extractions only about 20% of this activity remained associated with nuclear pellets. Sonication of nuclei with 1 M NaCl was ineffective in solubilizing this activity (data not shown).

TABLE II-5

Cumene Hydroperoxide Levels Following Incubations with 1 mM GSH at 37°C for 15 min: The Effects of Preincubations with Cystine, NEM and Heat.

Preincubation Conditions[a]	Concentration (mM)	Cumene Hydroperoxide following incubation[b] (mol)
Control[c]	-	0.40
Cystine	0.5	0.30
NEM	0.6	0.29
15 min at 60°C	-	0.39
No addition	-	N.D.[d]

[a] Preincubations consisted of exposing nuclear suspensions for 15 min to the designated treatment. Unless otherwise noted, all preincubations occurred at 37°C.

[b] Following preincubations, nuclei were pelleted and then resuspended in incubation buffer containing 1 mM GSH. Nuclear suspensions were incubated at 37°C for 15 min. At time zero, 0.80 umol cumene hydroperoxide were added.

[c] Control preincubation had no nuclei.

[d] Not detectable (less than 0.02 umol).

DISCUSSION

NADPH induced nuclear peroxidation was measured by monitoring the loss of unsaturated fatty acids and the formation of MDA. The results from these studies show that nuclear constituents are susceptible to lipid peroxidation. Analysis of fatty acid composition data indicated that the polyunsaturated arachidonic and docosahexaenoic acid fractions were primarily destroyed by peroxidation and that MDA is formed from unsaturated fatty acids containing three or more double bonds as previously reported (36). Since arachidonic and docosahexaenoic acid fractions both contain more than three double bonds, nuclear peroxidation primarily affects those fatty acids which can generate MDA. Also, GSH inhibited both MDA formation and loss of polyunsaturated fatty acids. Together, these data suggest that MDA formation adequately measures the loss of unsaturated fatty acids which occurs in NADPH induced nuclear peroxidation.

This is the first report that GSH can inhibit nuclear lipid peroxidation. The initial concentration of GSH determined the duration of the GSH induced lag in peroxidation with higher concentrations of GSH extending the lag prior to the rapid peroxidation of unsaturated fatty acids. These effects occurred at concentrations of GSH below those typically found intracellularly, which range from 0.5 to 10 mM (37), depending on the tissue examined.

The concentration effects of GSH described in this report agree well with the results obtained by Burk with microsomes (18). In this microsomal system, 0.1 mM GSH produced an 11 min lag prior to the onset of ascorbate initiated lipid peroxidation, and 1 mM GSH produced a 29 min lag (18). In the nuclear NADPH induced peroxidation system, 0.1 mM and 1 mM GSH provided delays of 15 and 30 min respectively.

Several lines of evidence suggest that a protein is involved in the GSH dependent inhibition of peroxidation. Exposure of isolated nuclei to heat (60°C) or trypsin yielded time dependent decreases in the protection afforded by GSH. Experiments also indicated that of the thiol compounds tested, only GSH produced a lag prior to peroxidation. Therefore, the GSH dependent inhibition of peroxidation is not due to a nonspecific thiol effect. Instead, the data suggest that substrate specificity may be involved which causes a GSH dependence. Specificity for GSH was also demonstrated by Burk in the inhibition of microsomal lipid peroxidation. No inhibition was seen when GSH was replaced by cysteine, DTT or β-mercaptoethanol in this system (18). The cytosolic protein investigated by Gibson et al. (20) had much broader substrate specificity requirements. Cysteine, DTT, and β-mercaptoethanol all inhibited the lipid peroxidation of heated microsomes when incubated in the presence of this cytosolic protein (20). The GSH dependent peroxidase

purified by Ursini et al. (19) inhibited liposomal lipid peroxidation with either β-mercaptoethanol or GSH. However, concentrations of GSH above 1 mM were required to inhibit liposomal lipid peroxidation (19).

The GSH dependent inhibition of lipid peroxidation was also affected by thiol modifying reagents and disulfides. Pretreatment with NEM abolished the protective effects of GSH. The disulfides GSSG, cystamine, and cystine produced similar inhibition but at varying concentrations. These data support the hypothesis that a labile protein is involved in the GSH dependent inhibition of peroxidation and also suggests that an essential sulfhydryl group is required for its activity. The activity of many enzymes containing an essential sulfhydryl group can be modified by treatment with disulfides (38). This inhibition is mediated through the formation of protein-mixed disulfides (38). The cytosolic protein described by Gibson et al. also possesses at least one sulfhydryl group required for its activity (20).

Another potent inhibitor of the GSH dependent protection against peroxidation is cumene hydroperoxide. However, the extent of this inhibition was greatly diminished if nuclei were pretreated with both cumene hydroperoxide and GSH. The concentration of cumene hydroperoxide required to abolish the GSH effect was increased ten fold when GSH was included in the pre-incubation mixture. This dramatic effect of GSH on cumene

hydroperoxide dependent inhibition can be explained by examining the hydroperoxide levels following preincubations. No cumene hydroperoxide was detected in those nuclear preincubations containing GSH. To a certain extent, the reduction of cumene hydroperoxide levels occurred due to non-enzymatic reactions with GSH. However, experiments determined that nuclei catalyze this reaction, and more specifically, that nuclei catalyze the reduction of cumene hydroperoxide to cumyl alcohol. Tests were conducted to determine whether this peroxidase activity was responsible for the GSH dependent inhibition of peroxidation. Pretreatment of nuclei with 0.5 mM cystine, 0.6 mM NEM, or exposure to 60°C temperatures all inhibited the loss of cumene hydroperoxide. As previously demonstrated, these treatments also affected the ability of GSH to protect against lipid peroxidation. Therefore the peroxidase activity associated with nuclei appears to be responsible for the GSH dependent inhibition of lipid peroxidation.

Gibson et al. (39) demonstrated that the classical enzyme glutathione peroxidase is unable to inhibit lipid peroxidation of microsomal membranes. These researchers concluded that this soluble peroxidase is unable to interact with lipid hydroperoxides while they remain associated with microsomal membranes (39). If, however, the glutathione peroxidase activity was associated with the phospholipid bilayer of the nuclear membrane, this

restriction may not apply. Such an association may contribute to the ability of the peroxidase to reduce lipid hydroperoxides. Since lipid hydroperoxides can initiate lipid peroxidation, the reduction of these compounds can contribute to the inhibition of peroxidation. Evidence suggests that the GSH dependent peroxidase purified by Ursini et al. is interfacial in character (21). Also, the protective effects of GSH described by Burk in microsomes were due to a microsomal protein (18).

Nuclear pellets were washed twice after isolation to remove any cytosolic contamination. Despite these procedures, the GSH dependent inhibition of peroxidation still remained associated with isolated nuclei. Extraction of nuclei with 1 M NaCl accompanied with brief sonication failed to solubilize the peroxidase activity. Extraction with 0.3% Triton X-100, however, solubilized the GSH dependent peroxidase activity (data not shown). Electron microscopy studies conducted by Dabeva et al. indicate that treatment with even higher concentrations of Triton X-100 removed the outer nuclear membrane but preserved the integrity of the remaining nucleus (40). Based on this information, it appears that the peroxidase activity is associated with the nuclear membrane. This activity in conjunction with GSH may contribute to the inhibition of lipid peroxidation in nuclear membranes and thereby preserve the integrity of this important membrane

system. Increasing evidence suggests that this inhibition of peroxidation may in turn protect the structure and function of DNA. Work is currently underway in this laboratory to purify the nuclear peroxidase and determine its ability to act on lipid hydroperoxides.

CONCLUSION

Rat liver nuclei were susceptible to both ferric-ADP/NADPH and ferric-ADP/ascorbate induced lipid peroxidation. Peroxidation was accompanied by the destruction of polyunsaturated fatty acids and MDA formation. Although other thiols were tested, only GSH inhibited nuclear lipid peroxidation. The data suggest that a GSH dependent peroxidase activity is responsible for this inhibition and that this enzyme activity is localized in the nuclear membrane.

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PARTIAL PURIFICATION FROM RAT LIVER NUCLEI OF A GLUTATHIONE TRANSFERASE: ROLE IN THE INHIBITION OF NUCLEAR LIPID PEROXIDATION

ABSTRACT

Glutathione protects isolated rat liver nuclei against lipid peroxidation. This protection was abolished by exposing isolated nuclei to the glutathione transferase inhibitor S-octylglutathione. S-Octylglutathione also inhibited nuclear glutathione transferase activity and glutathione peroxidase activity. A large percentage of the glutathione transferase activity associated with isolated nuclei was solubilized with 0.3% Triton X-100. Studies suggest that this treatment removes nuclear membranes while preserving the integrity of the remaining nucleus. The glutathione transferase activity associated with the nuclear membrane was partially purified by utilizing a S-hexyl-glutathione affinity column. The partially purified nuclear glutathione transferase exhibited glutathione peroxidase activity towards lipid hydroperoxides in solution. Reconstitution experiments suggest that this enzyme can also inhibit the peroxidation of phosphatidylcholine liposomes. The data from this present study indicate that a glutathione transferase associated with the nuclear membrane may contribute to glutathione dependent protection of isolated nuclei against lipid peroxidation.

INTRODUCTION

Glutathione (GSH) is thought to play an important role in protecting cells against oxidative stress. Several studies have shown that the addition of GSH protected microsomal incubations against both NADPH and ascorbate induced lipid peroxidation (1-3). The mechanism involved in this protection remains in doubt, but results suggest that a microsomal protein is required for the GSH dependent inhibition of lipid peroxidation (1).

The classical selenium dependent glutathione peroxidase (4) and certain glutathione transferases (5) have been shown to reduced lipid hydroperoxides to lipid alcohols with the concurrent oxidation of two molecules of GSH to oxidized glutathione (GSSG). Since lipid hydroperoxides have been shown to be involved in the propagation of lipid peroxidation (6), the reduction of these compounds would be expected to protect membranes against lipid peroxidation.

Belouqui and Cederbaum showed that the addition of GSH produced a forty percent reduction in NADPH induced peroxidation of rat liver microsomes (7). However, the addition of the soluble enzymes glutathione peroxidase, glutathione reductase and glutathione transferase together with GSH did not significantly increase the protective capacity of GSH in these incubations.

In a similar set of experiments conducted by Gibson

et al. (8), heat denatured hepatic microsomes initiated with ascorbate and ferric iron were not protected by the addition of soluble glutathione transferase or glutathione peroxidase fractions in the presence of GSH (8). In this system, little reduction of membrane lipid hydroperoxides was detected. If, however, the microsomal lipids were free in solution, rather than incorporated in membranes, considerable reduction of lipid hydroperoxides occurred. On the basis of these data, Gibson et al. concluded that lipid hydroperoxides must be free in solution if they are to be substrates for the soluble enzymes glutathione transferase and glutathione peroxidase. In this case, the lipophilic environment of membranes apparently restricts interactions between enzymes located in the aqueous phase and lipid hydroperoxides located in the lipid bilayer.

Lipid hydroperoxides located in biological membranes increase the potential for lipid peroxidation. If this potential is to be diminished these peroxides must be removed from the membrane environment or be reduced to lipid alcohols. The association of a glutathione dependent peroxidase with membranes may encourage the reduction of lipid hydroperoxides located within lipid bilayers.

Previous work in this laboratory has shown that GSH protects isolated rat liver nuclei against lipid peroxidation. Results suggest that a GSH dependent peroxidase activity associated with the nuclear membrane is

responsible for this protection (9).

The existence of a membrane associated peroxidase activity has been proposed by Ursini et al. (10). These researchers purified a selenium containing glutathione peroxidase from pig heart. Although the enzyme had a similar amino acid composition, differences in substrate specificity requirements indicate that the enzyme is distinct from the classical glutathione peroxidase. Phospholipid hydroperoxides incorporated into liposomes were reduced by this enzyme but not by the classical glutathione peroxidase. Ursini et al. concluded that this glutathione dependent peroxidase is interfacial in character. The addition of this membrane associated glutathione peroxidase as well as GSH inhibited the iron ascorbate induced peroxidation of phosphatidylcholine liposomes (11).

In addition to the peroxidase described by Ursini et al. (10), a membrane bound glutathione transferase possessing glutathione peroxidase activity has been purified from rat liver microsomes (12). The solubilized enzyme possesses a monomer molecular weight of 14,000 and has been shown to reduce cumene hydroperoxide. This enzyme is unique with respect to the soluble glutathione transferases in that it is activated by N-ethylmaleimide (NEM) (13). At present, no research has investigated whether this enzyme can inhibit lipid peroxidation.

The present study describes the partial purification

of a nuclear membrane associated glutathione transferase. This glutathione transferase possesses glutathione dependent peroxidase activity towards cumene hydroperoxide, tert-butyl hydroperoxide and lipid hydroperoxides in solution. Reconstitution experiments suggest that this enzyme can inhibit the peroxidation of liposomes.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats (250-300g) were used throughout the course of this study. These animals had free access to Purina Rat Chow and water.

Chemicals

NADPH (type-I), ADP (grade III), soybean L-α-phosphatidylcholine (type III-s), S-octylglutathione and S-hexylglutathione-agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Glutathione peroxidase (bovine erythrocytes) was purchased from Calbiochem-Behring (San Diego, CA). 10,13-Nonadecadienoic acid was obtained from Nu Chek Prep, Inc. (Elysian, MN). The enzymes lipoxidase (type I) and phospholipase A_2 (porcine pancreas) were also obtained from Sigma Chemical Co. All other chemicals were obtained from Sigma Chemical Co.

Isolation of Nuclei

Animals were anesthetized with ether then decapitated. Livers were immediately removed and placed on ice. Nuclei were isolated from rat liver according to Blobel and Potter (14) with slight modifications for large scale preparations utilizing a Beckman SW 28 rotor. Nuclei were resuspended in 30 ml 100 mM NaCl buffer TM[2] and centrifuged at 4000 x g for 10 min at 4°C. Nuclei were washed twice in this manner prior to all incubations

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[2]50 mM Tris, 5 mM $MgCl_2$ pH 7.5

to insure preparations were free of cytosolic contamination.

Nuclear Peroxidation Conditions

All incubations were conducted in 100 mM NaCl buffer TM at 37°C. The NADPH induced peroxidation system consisted of 1 mM NADPH, 1.7 mM ADP, 0.11 mM EDTA and 0.1 mM $FeCl_3$ (15). Peroxidation was initiated at time zero by the addition of 1.7 mM ADP, 0.11 mM EDTA and 0.1 mM $FeCl_3$. Zero time points were taken immediately after the addition of the ADP, EDTA and $FeCl_3$ solution.

Preparation of Lipid Hydroperoxides

The synthesis of 14-hydroperoxy-nonadecadienoic acid was performed according to the procedures of Hamberg and Samuelsson (16).

Preparation of Liposomes

Liposomes were prepared according to the procedures of Racker (17) with slight modifications. Soybean phosphatidylcholine was added to 100 mM NaCl TM buffer containing 1.6% cholate and 10% glycerol. Solutions were sonicated until clear. Following sonication, phospholipid solutions were dialyzed at 4°C against 500 volumes of 100 mM NaCl TM buffer containing 10% glycerol for 16 hrs. The dialysis buffer was sparged with argon, and all dialysis occurred under argon.

Proteins were reconstituted into liposomes by adding washed (ultrafiltration--Amicon YM-10) and concentrated protein solutions to phospholipid solutions containing

1.6% cholate and 10% glycerol dissolved in 100 mM NaCl TM buffer (45 mg phosphatidylcholine/mg protein). The protein solution was added following sonication and immediately prior to dialysis.

Peroxidation of Liposomes

Liposomes were resuspended in 100 mM NaCl buffer TM at 37°C at a concentration of 0.5 mM. The ascorbate peroxidation system consisted of 200 uM ascorbate, 50 uM ferric chloride and 2.5 mM ADP.

Peroxidation was initiated at the time zero by the addition of the ADP and iron solution. Zero time points were taken immediately following the addition of this solution. Liposomes were incubated in this manner for one hr at 37°C.

Phospholipase A_2 digestions of peroxidized liposomes consisted of incubating peroxidized liposomes with 4 units phospholipase A_2 and 1 mM $CaCl_2$ at 37°C for 15 min.

Enzyme Assays

Glutathione transferase activity was assayed according to the procedures of Habig et al. (18) with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). Glutathione peroxidase activity towards cumene hydroperoxide, tert-butyl hydroperoxide (1.5 mM) and hydrogen peroxide were measured as described by Lawrence and Burk (19). Glutathione peroxidase activity measurements towards 14-hydroperoxy-nonadecadienoic acid and peroxidized liposomes were conducted as described above except that both

substrates were present at concentrations of 0.1 mM. 14-Hydroperoxy-nonadecadienoic acid was dissolved in 70% ethanol prior to glutathione peroxidase assays.

Chemical Assays

Proteins were determined by the method of Lowry et al. (20) as described by Peterson (21). Malondialdehyde (MDA) levels in nuclear incubations were determined by using the thiobarbituric acid assay as developed by Wills (22) with slight modifications. Aliquots (0.25 ml) of nuclear suspensions were added to 0.5 ml 10% (w/v) trichloroacetic acid. The resulting mixture was chilled on ice then centrifuged for 2 min at 15,000 x g. Following centrifugation, supernatants were removed and added to 1.0 ml of 0.67% (w/v) thiobarbituric acid. Samples were heated at 95°C for 20 min and then cooled to room temperature. Absorbance values were measured at 532 nm.

Malondialdehyde levels in liposomal incubations were assayed according to the procedures of Buege and Aust (23).

RESULTS

GSH Dependent Metabolism of Hydroperoxides by Isolated Rat Liver Nuclei

Isolated nuclei were assayed for glutathione peroxidase activity with cumene hydroperoxide, hydrogen peroxide and tert-butyl hydroperoxide. The specific activity measurements determined for these compounds are shown in Table III-1. No glutathione peroxidase activity was detected when isolated nuclei were incubated with hydrogen peroxide, but glutathione peroxidase activity was detected in incubations containing cumene hydroperoxide or tert-butyl hydroperoxide.

Effects of S-Octylglutathione on the Glutathione Dependent Protection of Isolated Nuclei Against Lipid Peroxidation

Glutathione has been shown to protect isolated nuclei against lipid peroxidation (9). The addition of 1 mM GSH produced a thirty minute lag period prior to the onset of NADPH induced lipid peroxidation. Incubations containing no GSH exhibited a lag of only 5 min before rapid peroxidation ensued. The effects of the glutathione transferase inhibitor, S-octylglutathione (24), on this glutathione dependent protection are shown in Figure III-1. The GSH dependent lag period was abolished when isolated nuclei were incubated with 1 mM GSH and 0.2 mM S-octylglutathione. In separate experiments, S-octylglutathione inhibited both glutathione transferase activity and

TABLE III-1

Glutathione Dependent Peroxidase Activity of Isolated Nuclei: Specific Activity Measurements

Hydroperoxide	Specific Activity $\frac{\text{milliunits}^a}{\text{mg protein}}$
Cumene Hydroperoxide	8.2
tert-Butyl Hydroperoxide	20.0
Hydrogen Peroxide	N.D.[b]

[a] Milliunits of glutathione peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1 nmol GSH per min at 25°C.

[b] Not detectable (less than 1 milliunit per mg protein)

Figure III-1. Effects of S-octylglutathione on the GSH dependent inhibition of NADPH induced nuclear lipid peroxidation. (■)- no GSH, (○)- 1 mM GSH and (△)- 1 mM GSH and 0.2 mM S-octylglutathione. Nuclei were pre-incubated with NADPH and the indicated additions for 10 min prior to the initiation of peroxidation. The incubation conditions and the NADPH peroxidation system are described in the experimental procedures section.

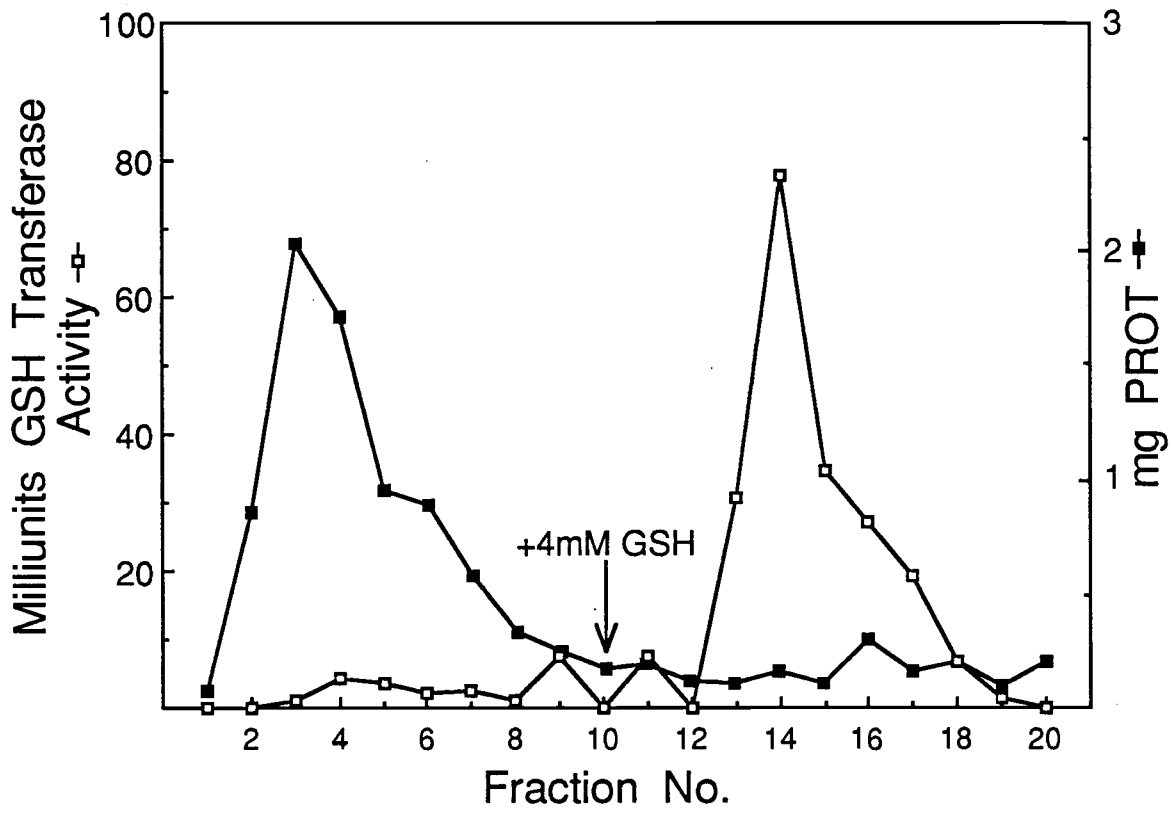
Milliunits GSH Transferase Activity
mg PROT
100
80
60
40
20
3
2
1
+4mM GSH
2
4
6
8
10
12
14
16
18
20
Fraction No.

Figure III-1

glutathione peroxidase activity (as measured by cumene hydroperoxide reduction) in isolated nuclei preparations (data not shown).

Partial Purification of Nuclear Glutathione Transferase

Isolated nuclei were resuspended in 30 ml NaCl TM buffer and centrifuged at 4000 x g for 10 min at 4°C. Nuclei were washed twice in this manner prior to further purification procedures. Glutathione transferase activity was solubilized from nuclei by resuspending nuclear pellets in 8 ml (approximately 25 mg protein per ml detergent solution) 0.3% Triton 100 mM NaCl TM buffer containing 10% glycerol and 1 mM EDTA. Nuclear suspensions were then centrifuged at 15,000 x g for 2 min. The resulting supernatant was applied to a 10 ml S-hexyl-glutathione-agarose affinity column. Following application of the solubilized nuclei, the affinity column was washed with forty one ml of 100 mM NaCl TM buffer containing 10% glycerol and 1 mM EDTA. Glutathione transferase activity was eluted from the affinity column with 100 mM NaCl TM buffer pH 9.5 containing 10% glycerol, 1 mM EDTA and 4 mM GSH at a flow rate of 0.9 ml/min. Shortly after the addition of the elution buffer (fraction 12), the flow was stopped for a 15 min period.

Figure III-2 shows the distribution of protein and glutathione transferase activity in the 5 ml fractions collected from the S-hexylglutathione affinity column. As this figure shows, the majority of the glutathione trans-

Figure III-2. Partial purification of nuclear glutathione transferase--S-hexylglutathione affinity column. Milli-units glutathione transferase (CDNB) and mg protein are plotted verses the number of column fractions collected. Fractions were 5 ml in volume. Following the application of detergent solubilized nuclei, the column was eluted with 100 mM NaCl TM buffer containing 10% glycerol and 1 mM EDTA (fractions 1-9). At the start of fraction 10, the column was eluted with 100 mM NaCl TM buffer pH 9.5 containing 10% glycerol, 1 mM EDTA and 4 mM GSH at a flow rate of 0.9 ml/min. The flow rate was interrupted at the beginning of fraction 12 for 15 min to allow the column to equilibrate with the new elution buffer.

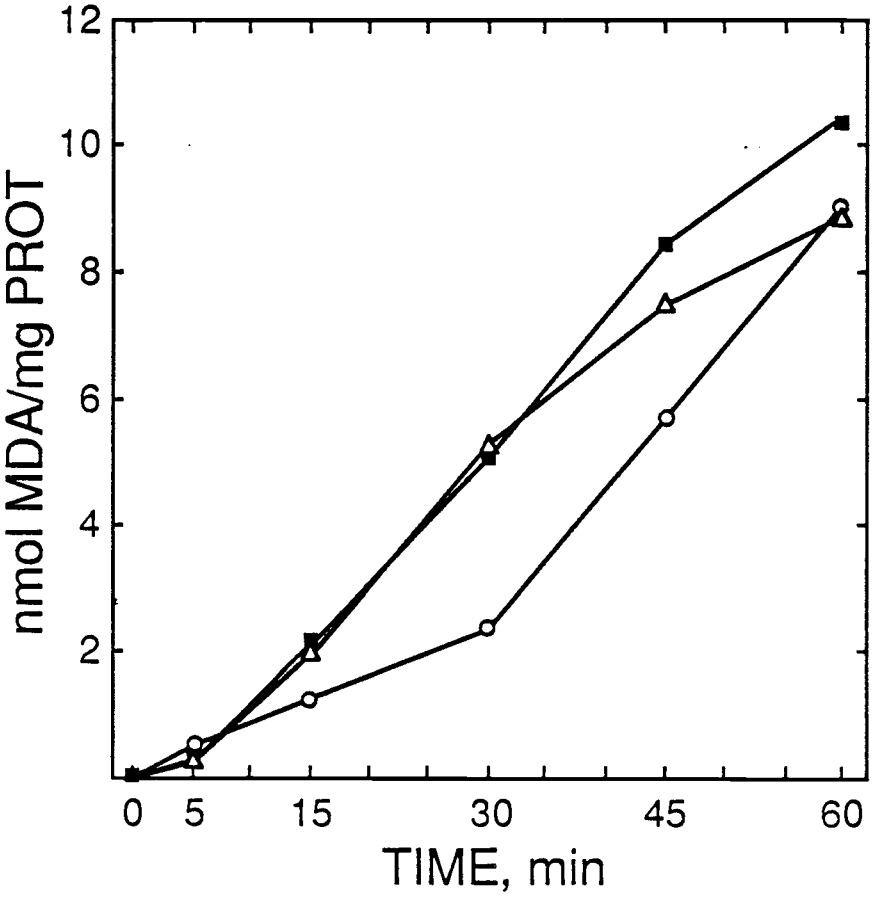

Figure III-2

ferase activity applied is retained by the affinity column and eluted with 4 mM GSH. Fractions 13 through 18 were pooled and concentrated for further analysis of the partially purified protein. SDS gel electrophoresis of this partially purified fraction treated with β-mercaptoethanol revealed 6 bands. No bands were detected below a molecular weight of 21,500 (data not shown).

The yield and extent of purification of the nuclear glutathione transferase obtained from these procedures are reported in Table III-2. The nuclear glutathione transferase activity was purified 72.5 fold and about 35% of the original activity found in isolated nuclei was recovered.

Glutathione Dependent Peroxidase Activity of Partially Purified Nuclear Glutathione Transferase

The partially purified nuclear glutathione transferase was assayed for glutathione dependent peroxidase activity towards a variety of hydroperoxides (Table III-3). No activity was exhibited by the enzyme towards hydrogen peroxide. However, both cumene hydroperoxide and tert-butyl hydroperoxide were determined to be substrates for the enzyme. The partially purified enzyme was also incubated with the fatty acid 14-hydroperoxy-nonadecadienoic acid to determine if lipid hydroperoxides were suitable substrates for the enzyme. As Table III-3 shows, a specific activity of 0.354 units per mg protein was measured for the enzyme towards 14-hydroperoxy-

TABLE III-2

Partial Purification of Nuclear Glutathione Transferase

	Yield (%)	Specific Activity $\frac{\text{milliunits}^a}{\text{mg prot}}$	Purification Factor
Whole Nuclei	100.0	9.4	---
Detergent Solubilized Fraction[b]	70.9	68.3	7.3x
Affinity Column[c]	35.6	679.0	72.5x

[a] Milliunits of glutathione peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1 nmol GSH per min at 25°C.

[b] Nuclei were mixed with 0.3% Triton 100 mM NaCl TM buffer containing 10% glycerol and 1 mM EDTA. Nuclear suspensions were then centrifuged at 15,000 x g for 2 min. Supernantants were retained for glutathione transferase assays.

[c] S-hexylglutathione-agarose affinity column

TABLE III-3

Glutathione Dependent Peroxidase Activity of Partially Purified Nuclear Glutathione Transferase

Hydroperoxide	Specific Activity units[a]/mg protein
hydrogen peroxide	N.D.[b]
cumene hydroperoxide	0.567
lipid hydroperoxide[c]	0.354
tert-butyl hydroperoxide	0.080

[a] Units of glutathione peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1 umol GSH per min at 25°C.

[b] Not detectable (less than 0.028 units per mg protein)

[c] 14-hydroperoxy-nonadecadienoic acid

nonadecadienoic acid. Preincubation of the enzyme with 1 mM NEM or 0.2 mM S-octylglutathione inhibited the GSH dependent reduction of cumene hydroperoxide (data not shown).

Glutathione Dependent Peroxidase Activity of Partially Purified Nuclear Glutathione Transferase Towards Peroxidized Liposomes

Experiments were conducted to assess the relative abilities of the partially purified nuclear glutathione transferase and the classical selenium containing glutathione peroxidase to reduce lipid hydroperoxides still associated with liposomal membranes (Table III-4). Although both enzymes exhibited activity towards cumene hydroperoxide, only slight activity was measured towards peroxidized liposomes. Exposure of the peroxidized liposomes to the enzyme phospholipase A_2, increased the glutathione dependent peroxidase activity of both enzymes. Phospholipase A_2 treatment increased the activity of the selenium containing glutathione peroxidase seven fold and tripled the activity of the nuclear glutathione transferase towards the peroxidized liposomes.

Capacity of Nuclear Glutathione Transferase Reconstituted into Liposomes to Inhibit Peroxidation

The addition of 1 mM GSH failed to protect phosphatidylcholine liposomes against lipid peroxidation during the course of a one hr incubation at 37°C. However, in several experiments, liposomes reconstituted with the

TABLE III-4

Glutathione Dependent Peroxidase Activity of Nuclear Glutathione Transferase Towards Peroxidized Liposomes

Substrate	Nuclear Glutathione Transferase Milliunits[b]	Selenium Containing Glutathione Peroxidase[a] Milliunits[b]
Cumene Hydroperoxide	1.0	1.2
Peroxidized Liposomes[c]	0.1	0.1
Peroxidized Liposomes +Phospholipase A_2[d]	0.3	0.7

[a] Selenium containing glutathione peroxidase purchased from Calbiochem.

[b] Milliunits of glutathione peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1 nmol GSH per min at 25°C.

[c] Liposomes were peroxidized according to the procedures described in the experimental procedures section.

[d] Peroxidized liposomes were incubated with 4 units phospholipase A_2 and 1 mM $CaCl_2$ for 15 min at 37°C.

partially purified nuclear glutathione transferase showed some protection against lipid peroxidation in the presence of 1 mM GSH (data not shown). The rates of peroxidation in liposomes varied widely from one experiment to another although the initiation system was not changed. Further experimentation is required to establish the conditions required to maintain the activity of the protein and produce liposomes that yield consistent rates of peroxidation.

DISCUSSION

Isolated nuclei catalyzed the GSH dependent reduction of tert-butyl hydroperoxide and cumene hydroperoxide but failed to metabolize hydrogen peroxide. This substrate specificity pattern is consistent with those exhibited by the glutathione transferases (25) (19). Previous studies in this laboratory have shown that GSH can inhibit the peroxidation of isolated nuclei and that a nuclear glutathione peroxidase activity is responsible for this protection (9).

The effects of the glutathione transferase inhibitor S-octylglutathione (24) on the capacity of GSH to inhibit nuclear peroxidation were investigated. Results indicate that incubation of isolated nuclei with S-octylglutathione abolished the GSH dependent inhibition of peroxidation. In addition, S-octylglutathione inhibited the conjugation of CDNB with GSH (glutathione transferase activity) and the GSH dependent reduction of cumene hydroperoxide (glutathione peroxidase activity) catalyzed by isolated nuclei. These data provide strong evidence that a nuclear glutathione transferase is responsible for the GSH dependent inhibition of nuclear lipid peroxidation.

Approximately seventy percent of the nuclear glutathione transferase activity (as measured by CDNB conjugation) was solubilized by treating isolated nuclei with 0.3% Triton X-100. In experiments conducted by Dabeva et

al. (26), treatment with similar concentrations of Triton X-100 altered nuclear membrane structure while preserving the integrity of the remaining nucleus. Thus, these data suggest that a large fraction of nuclear glutathione transferase activity is associated with the nuclear membrane.

Partial purification of this detergent solubilized glutathione transferase activity was achieved with a S-hexylglutathione affinity column. Following these procedures, specific activity measurements were conducted with the partially purified fraction and a variety of substrates. Activity was detected towards CDNB, cumene hydroperoxide, lipid hydroperoxides, and tert-butyl hydroperoxide. However, no activity was detected towards hydrogen peroxide.

Ursini et al. (10) recently purified an interfacial glutathione peroxidase. This enzyme has been shown to reduce linoleic acid hydroperoxides, cumene hydroperoxide, tert-butyl hydroperoxide and hydrogen peroxide. However, this enzyme does not conjugate CDNB with GSH (11). Substrate specificities indicate that this enzyme is distinct from the nuclear glutathione transferase.

A microsomal glutathione transferase has also been purified which exhibits glutathione dependent peroxidase activity toward cumene hydroperoxide (12). This enzyme is unique with respect to the other glutathione transferases in that it is activated by prior treatment with NEM and

has a molecular weight of 14,000. Although small amounts of this enzyme have been reported to be associated with nuclear fractions (27), it is not the same enzyme as the partially purified nuclear glutathione transferase described in this study. NEM inhibited rather than activated this nuclear enzyme and no protein was detected below a molecular weight of 21,500 when the partially purified nuclear GSH transferase was analyzed by SDS polyacrylamide gel electrophoresis.

Only slight glutathione dependent peroxidase activity was detected when the nuclear glutathione transferase fraction or the selenium containing glutathione peroxidase was incubated with peroxidized liposomes. However, this activity was increased by prior treatment of the peroxidized liposomes with phospholipase A_2. In the case of the selenium containing glutathione peroxidase, phospholipase A_2 treatment increased the glutathione dependent peroxidase activity seven fold, whereas phospholipase A_2 treatment increased the activity of nuclear glutathione transferase three fold. The results with the selenium containing glutathione peroxidase agree with those seen by Sevanian et al. (28). These researchers concluded that the selenium containing glutathione peroxidase could not efficiently interact with lipid hydroperoxides incorporated into liposomes. Phospholipase A_2 treatment releases these lipid hydroperoxides from the liposomal membrane and allows the subsequent interaction of these

compounds with the enzyme.

The nuclear glutathione transferase reduces lipid hydroperoxides in solution, but only low glutathione dependent peroxidase activities were seen when peroxidized liposomes were incubated with the enzyme. It may be that the enzyme must be incorporated into membranes in order to act on lipid hydroperoxides still associated with the hydrophobic environment of the membrane. Experiments suggest that the partially purified glutathione transferase can inhibit the peroxidation of liposomes if it is reconstituted into liposomal membranes. Thus, some reduction of lipid hydroperoxides still associated with the membrane environment by the enzyme must occur.

Further research is required to assess the role of the enzyme phospholipase A_2 in the process of the inhibition of nuclear lipid peroxidation. Phospholipase A_2 may increase the efficiency of the nuclear glutathione transferase to reduce lipid hydroperoxides by removing these substrates from the membrane environment. In so doing, it may also play an important role in inhibiting nuclear lipid peroxidation.

Our findings suggest that a glutathione transferase associated with the nuclear membrane can utilize GSH to reduce lipid hydroperoxides. Since lipid hydroperoxides have been shown to be involved in the propagation of lipid peroxidation, this enzyme may be responsible for the GSH dependent protection of nuclear membranes against lipid

peroxidation and the protection of DNA against lipid peroxidation damage.

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THE EFFECTS OF GLUTATHIONE ON THE α-TOCOPHEROL DEPENDENT INHIBITION OF NUCLEAR LIPID PEROXIDATION

ABSTRACT

Endogenous α-tocopherol levels in isolated rat liver nuclei were determined to be 0.045 mol% (based on phospholipid content). This value corresponds to 967 polyunsaturated fatty acid moieties to one molecule of α-tocopherol in the nuclear membrane. Isolated nuclei incubated with various concentrations of exogenous α-tocopherol took up less than 20 percent of initial levels of α-tocopherol present in the incubation media. Exogenous α-tocopherol incorporated in isolated nuclei above a threshold value of 0.085 mol% effectively inhibited NADPH induced lipid peroxidation. The addition of 1 mM glutathione lowered the threshold levels of α-tocopherol needed to inhibit lipid peroxidation to about 0.040 mol%. The data suggest that glutathione and α-tocopherol act synergistically to inhibit nuclear lipid peroxidation. We propose that glutathione can act independently of α-tocopherol as a substrate for nuclear peroxidase activity to aid in the maintenance of a threshold level of α-tocopherol needed for protection against lipid peroxidation.

INTRODUCTION

Vitamin E is believed to function as an important cellular antioxidant. α-Tocopherol (α-TH), a major component of vitamin E, inhibits in vitro lipid peroxidation in a variety of membrane systems (1-3). Evidence suggests that α-TH exerts its antioxidant activity by scavenging free radicals generated in lipid peroxidation (4). Previous studies conducted in this laboratory have shown that α-TH incorporated in soybean phosphatidylcholine liposomes inhibited iron induced lipid peroxidation. Protection against peroxidation occurred only if α-TH levels were incorporated into liposomes above a certain critical level (5). Similar results were reported by Fukazawa et al. (6) utilizing egg lecithin liposomes. α-Tocopherol incorporated in liposomes above a threshold value of 2 nmol/mmol egg lecithin completely inhibited lipid peroxidation while subthreshold concentrations of α-TH failed to protect against peroxidation.

In addition to the findings with vitamin E, several studies have demonstrated that glutathione (GSH) can inhibit microsomal lipid peroxidation (7-9). The addition of GSH produced a lag period prior to the onset of microsomal lipid peroxidation in these studies. Following the cessation of this lag period, lipid peroxidation ensued at a rate characteristic of incubations lacking GSH (7). Although the mechanism involved in this GSH dependent

inhibition remains in doubt, several lines of evidence suggest that a microsomal protein is required for protection by GSH (7, 8).

Much the same results were obtained from experiments conducted in this laboratory with rat liver isolated nuclei and GSH (10). Glutathione protected isolated nuclei from lipid peroxidation by inducing a lag period prior to peroxidation. Evidence obtained form these experiments suggest that a nuclear membrane protein is involved in this GSH dependent protection and that this protein possesses a GSH dependent peroxidase activity (10).

The following study examines the effects of GSH on the ability of α-TH to inhibit nuclear lipid peroxidation. Nuclear α-TH levels were augmented by preincubating isolated nuclei with exogenous α-TH. Following these preincubations, the levels of α-TH required to inhibit lipid peroxidation were determined. The data indicates that GSH acts synergistically with α-TH to protect against lipid peroxidation.

EXPERIMENTAL PROCEDURES

Chemicals

Glutathione, NADPH (type I) and ADP were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol and γ-tocopherol were obtained from Eastman Kodak Co. (Rochester, NY).

Animals

Male Sprague Dawley rats (Simonsen Labs., Gilroy, CA) (250 -300 g) were used throughout the course of this study. Animals had free access to Purina Rat Chow and water.

Isolation of Rat Liver Nuclei and Preincubation with α-Tocopherol

Rats were anesthetized with ether then decapitated. Livers were immediately removed and placed on ice. Nuclei were isolated from rat liver according to Blobel and Potter (11) with slight modifications for large scale preparations utilizing a Beckman SW 28 rotor. After isolation, nuclear pellets were resuspended in 10 ml 100 mM NaCl buffer TM[3]. Various concentrations of α-TH were dissolved in absolute ethanol and added to nuclear suspensions so that the final concentration of ethanol was 1 percent (v/v). Nuclear suspensions containing α-TH were incubated at 37°C for fifteen min with swirling. Nuclei were pelleted after incubation by centrifuging at 4000 x g

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[3]50 mM Tris, 5 mM $MgCl_2$ pH 7.5

for ten min at 4°C. Nuclei were washed with 20 ml 100 mM NaCl buffer TM to remove unincorporated α-TH.

Peroxidation of Isolated Nuclei

Nuclear pellets containing added α-TH were resuspended in 100 mM NaCl buffer TM at 37°C. The peroxidation system consisted of 1 mM NADPH, 1.7 mM ADP, 0.11 mM EDTA and 0.1 mM $FeCl_3$ (12). Peroxidation was initiated at time zero by the addition of the ADP, EDTA and $FeCl_3$ solution.

Biochemical Determinations

α-Tocopherol levels were measured by reverse phase HPLC with fluorescence detection as described by Fariss et al. (13) with slight modifications. γ-Tocopherol, an internal standard, was added to nuclear suspensions according to the procedures of Fariss et al. (13) prior to extraction with hexane (14). Hexane extracts were evaporated under nitrogen, and the residue was dissolved in methanol. γ-Tocopherol and α-TH were eluted isocratically from a spherisorb ODS-II column (4.6 x 260 mm) (Alltech, Waukegan, IL) with 95 percent methanol/water (v/v) at a flow rate of 1.7 ml/min.

Proteins were determined according to the procedures of Lowry et al. (15) as modified by Peterson (16). Phosphorus levels were measured using the procedures of Fiske and Subbarow (17) as modified by Bartlett (18).

Malondialdehyde (MDA) levels were determined by using the thiobarbituric acid assay as developed by Wills (19)

with the following modifications. Aliquots (0.25 ml) of nuclear suspensions were added to 0.5 ml 10 percent (w/v) trichloroacetic acid. The resulting mixture was cooled to 4°C then centrifuged for 2 min at 15,000 x g. Supernatants were decanted off and added to 1.0 ml of 0.56 percent (w/v) thiobarbituric acid. Samples were heated at 95°C for twenty min and then cooled to room temperature. Absorbance values were measured at 532 nm.

RESULTS

Endogenous levels of α-TH measured in isolated rat liver nuclei are shown in Table IV-1. A value of 0.039 nmol α-TH per mg protein was obtained. Since a certain amount of autoxidation of α-TH may occur during nuclear isolation, this value represents a minimum value for endogenous levels of nuclear α-TH. However, when butylated hydroxyanisole was included in the isolation media to reduce the autoxidation of α-TH, the amount of α-TH detected in nuclei was not significantly increased (data not shown). A value of 0.045 mol percent α-TH (based on total mol phospholipid as determined by phosphate measurements) was calculated from a conversion factor of 86.7 nmol phospholipid per mg protein.

A series of incubations were performed to augment the levels of α-TH incorporated in isolated nuclei. Nuclei were exposed to various concentrations of α-TH then pelleted and washed. Figure IV-1 shows the percent of initial levels of α-TH added to incubations which remained associated with nuclei following these incubations and washings. At most, only about 20% of initial levels of α-tocopherol were incorporated into isolated nuclei.

Experiments utilizing nuclei supplemented with α-TH were conducted to assess the ability of exogenous α-TH to protect against lipid peroxidation (as measured by MDA formation). The time course of α-TH depletion and the

TABLE IV-1

Endogenous Levels of α-TH in Isolated Rat Liver Nuclei

$\frac{\text{nmol } \alpha\text{-TH}}{\text{mg Prot}}$	mol % α-TH[a]	$\frac{\text{nmol PUFA}}{\text{nmol } \alpha\text{-TH}}$[b]
0.039 ± 0.009 (9)[c]	0.045	967

a (mol α-TH / mol phospholipid) x 100

b Based on 43.5 percent of total moles fatty acids are polyunsaturated fatty acids (PUFA) (10).

c Mean values ± S.E. Number of observations in parentheses.

Figure IV-1. The uptake of α-TH by isolated rat liver nuclei. Isolated nuclei were preincubated with various concentrations of α-TH for 15 min at 37°C with swirling. Following preincubations nuclei, were pelleted at 4000 x g for 10 min at 4°C. Nuclei were then washed to remove unincorporated α-TH and assayed for α-TH as described in the experimental procedures section. All values plotted in Figure IV-1 were corrected for endogenous levels of α-TH.

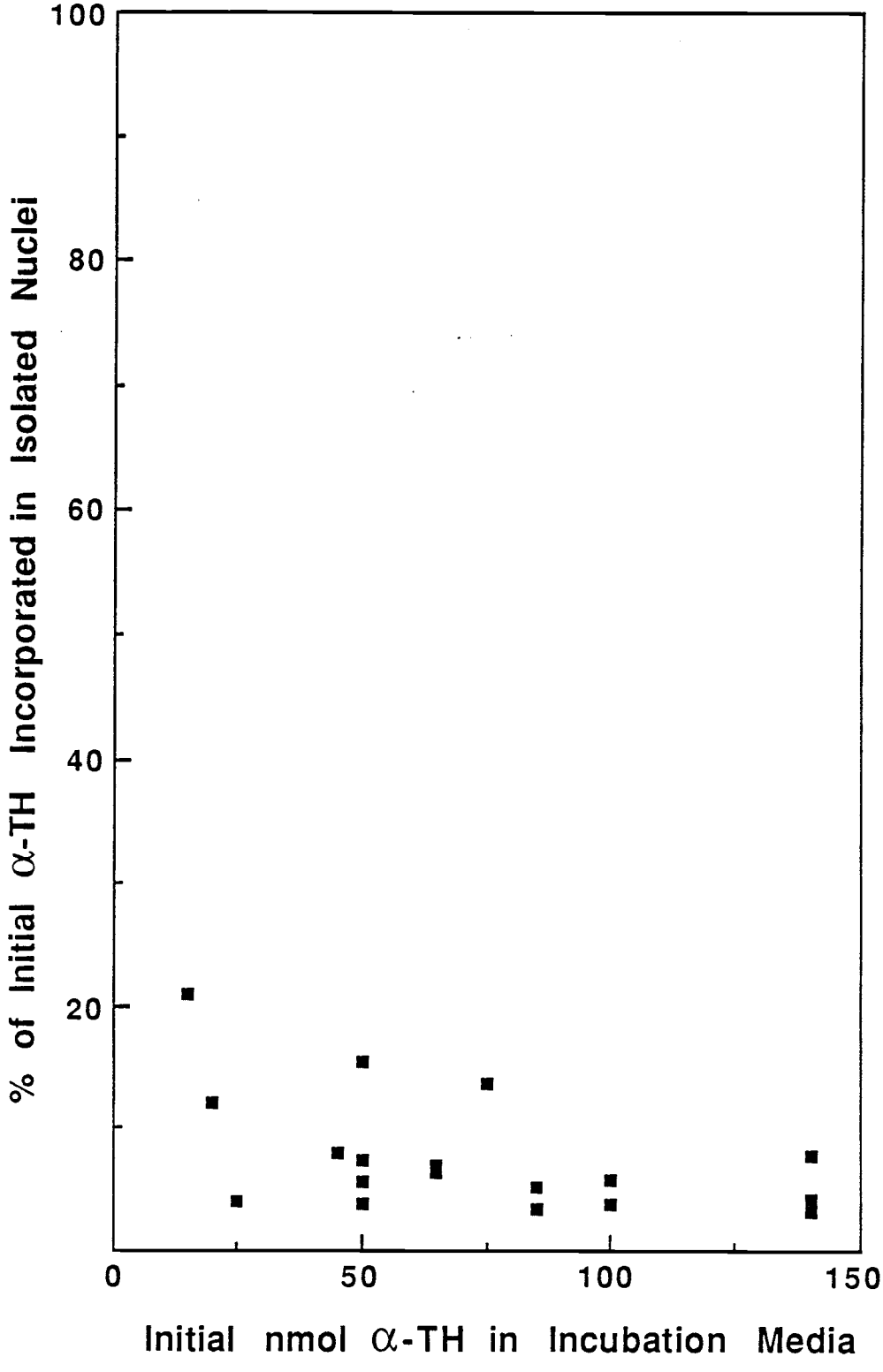
100
80
60
40
20
% of Initial α-TH Incorporated in Isolated Nuclei
0
50
100
150
Initial nmol α-TH in Incubation Media

Figure IV-1

corresponding MDA formation occurring following exposure of supplemented nuclei to peroxidizing conditions is reported in Table IV-2. Nuclei supplemented with α-TH exhibited much lower levels of MDA formation during the 60 minute incubation period than those measured in control nuclei. Little loss of α-TH was detected in nuclei supplemented with high α-TH levels.

The effects of GSH on the ability of exogenous α-TH to protect against lipid peroxidation are shown in Table IV-3. The addition of GSH inhibited the loss of α-TH and the formation of MDA. Nuclei supplemented with 0.107 mol percent α-TH exhibited a 30 minute lag period prior to the onset of lipid peroxidation. Nuclei containing similar levels of exogenous α-TH as well as GSH displayed only low levels of MDA formation during the incubation period and little loss of α-TH levels.

The results of several time course experiments (as shown in Tables IV-1 and IV-2) were compiled to determine what levels of α-TH are required to inhibit lipid peroxidation. Endogenous levels of α-TH in nuclei were altered by preincubating nuclear suspensions with varying amounts of exogenous α-TH. Nuclei were then washed and exposed to peroxidizing conditions as previously described. Zero time points were not included in these plots. The pooled results from experiments in which no GSH was added are displayed in Figure IV-2. Above 0.085 mol percent α-TH, little to no lipid peroxidation was detected. However,

TABLE IV-2

Time Course of α-TH Depletion and Induced Lipid Peroxidation in Isolated Rat Liver Nuclei

Time (min)	α-TH Supplemented Nuclei[a] α-TH (mol%)	α-TH Supplemented Nuclei[a] nmol MDA mg Prot	Control Nuclei α-TH (mol%)	Control Nuclei nmol MDA mg Prot
0	0.290	0.05	0.045	0.04
15	0.241	0.19	N.D.[b]	3.52
30	0.279	0.21	N.D.	6.50
45	0.244	0.27	N.D.	8.29
60	0.265	0.28	N.D.	9.74

[a] Nuclei were supplemented with α-TH according to the methods in the experimental procedures section.

[b] Not detectable

TABLE IV-3

The Effects of GSH on the Time Course of α-TH Depletion and Induced Lipid Peroxidation in Isolated Rat Liver Nuclei Supplemented with α-TH

	-GSH		+GSH[a]	
Time (min)	α-TH (mol %)	nmol MDA / mg Prot	α-TH (mol %)	nmol MDA / mg Prot
0	0.107	0.08	0.099	0.17
15	0.118	0.37	0.082	0.23
30	0.087	1.15	0.078	0.22
45	0.034	3.72	0.077	0.23
60	N.D.[b]	6.51	0.099	0.28

[a] 1 mM GSH

[b] Not detectable

Figure IV-2. The levels of α-TH required to inhibit NADPH induced nuclear peroxidation. Isolated nuclei were supplemented with various concentrations of exogenous α-TH then subjected to NADPH induced lipid peroxidation as described in the experimental procedures section. These results are a compilation of a series of time course experiments as shown in Tables IV-2 and IV-3. Zero time points were not included in this plot.

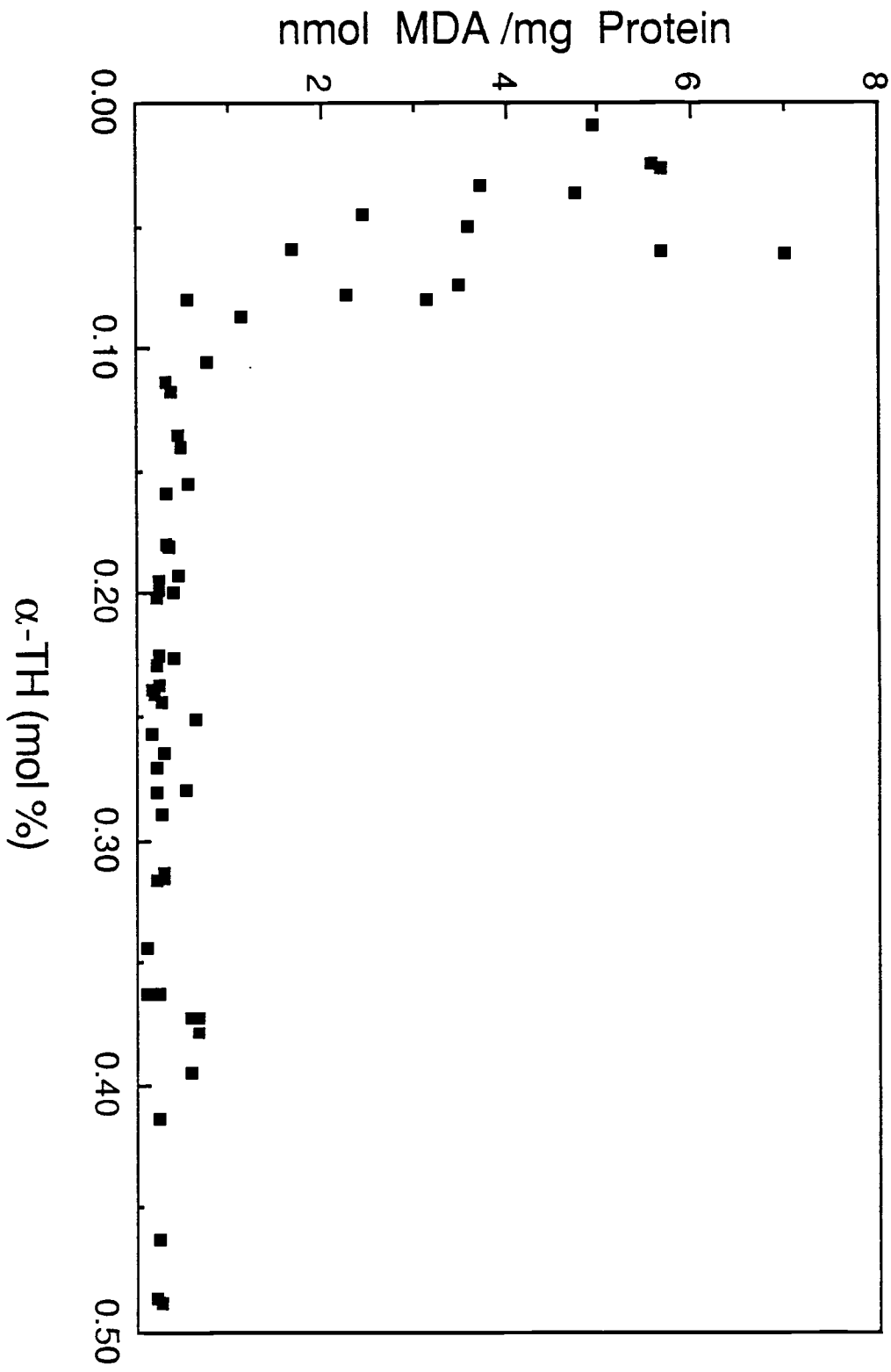

Figure IV-2

below this value, progressively higher values of MDA were formed. The sharp inflection point or threshold value at which α-TH becomes ineffective in inhibiting lipid peroxidation is clearly demonstrated by examining the percent of data points yielding values above 1 nmol MDA per mg protein. Above 0.100 mol percent α-TH, zero percent of the data points were above 1 nmol MDA per mg protein; below 0.079 mol percent α-TH, 100 percent of the data points were above 1 nmol MDA per mg protein.

Figure IV-3 shows the results of experiments in which 1 mM GSH was included in the incubation media. Incubation and peroxidation conditions were the same as those shown in Figure IV-2 with the exception of the addition of GSH. The threshold value determined from these data was about 0.04 mol percent α-TH. In these experiments, the addition of GSH increased the effectiveness of α-TH by lowering the levels of α-TH required for complete protection of nuclei against lipid peroxidation. Above 0.060 mol percent α-TH, 0 percent of the data points were above 1 nmol MDA per mg protein; below 0.019 mol percent, 100 percent of the data points were above 1 nmol per mg protein.

The levels of PUFA protected by α-TH are reported in Table IV-4. Values obtained from experiments conducted with egg phosphatidylcholine liposomes (5) were included for comparison. Results indicate that α-TH can protect about five times more molecules of PUFA in nuclear

Figure IV-3. The effects of GSH on the levels of α-TH required to inhibit NADPH induced nuclear peroxidation. Isolated nuclei were supplemented with various concentrations of exogenous α-TH then subjected to NADPH induced lipid peroxidation as described in the experimental procedures section. These results are a compilation of a series of time course experiments as shown in Tables IV-2 and IV-3. All incubations contained 1 mM GSH. Zero time points were not included in this plot.

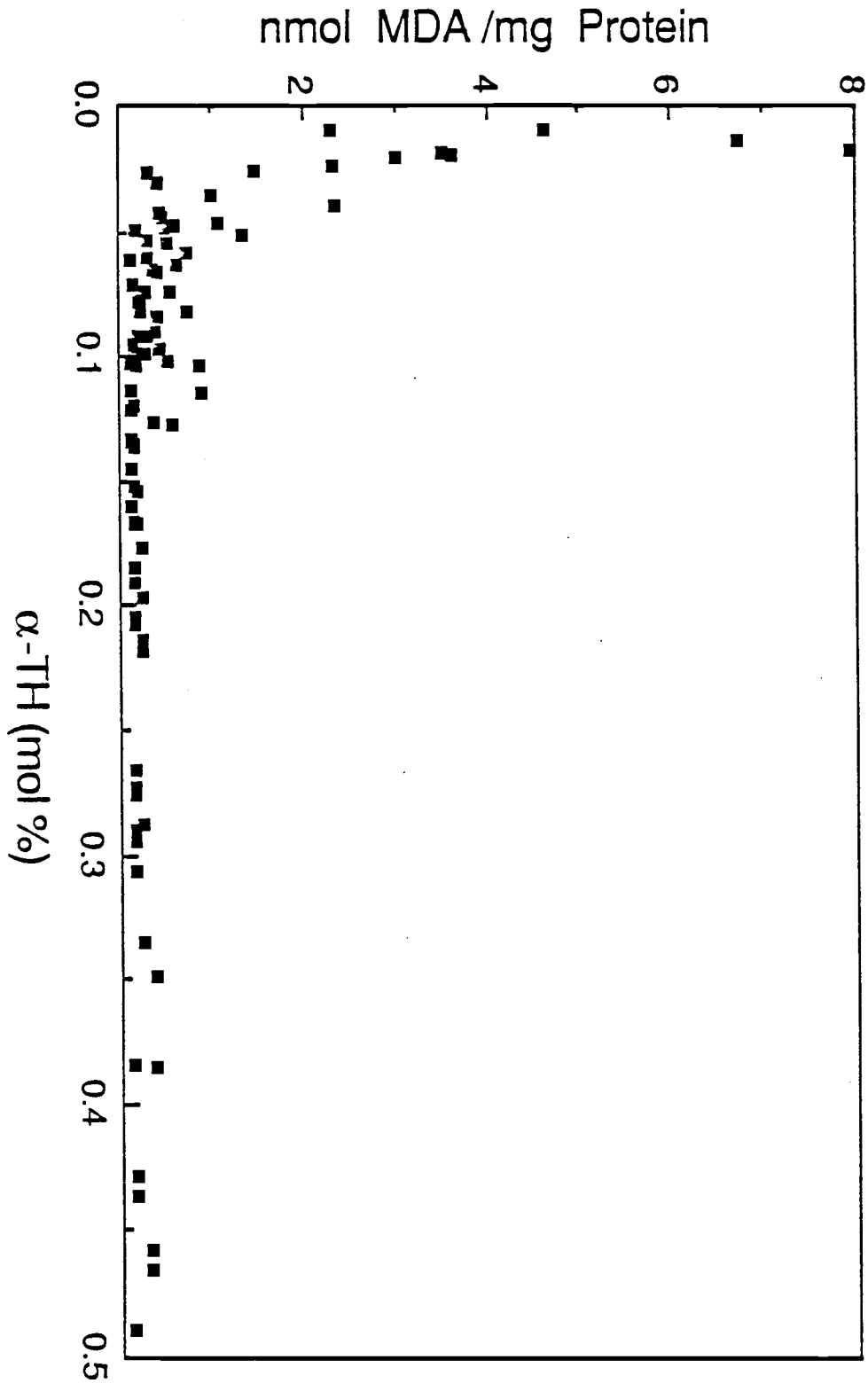

Figure IV-3

TABLE IV-4

Levels of α-TH Required to Protect Polyunsaturated Fatty Acids (PUFA) From Lipid Peroxidation

	nmol PUFA/nmol α-TH
Isolated Nuclei	512[a]
Isolated Nuclei + 1 mM GSH	1,088[a]
Egg Phosphatidylcholine Liposomes	104[b]

[a] Based on 43.5 % of total moles fatty acids are polyunsaturated fatty acids (10).

[b] Personal communication Dr. Daniel C. Liebler

membranes than in liposomal membranes. If GSH is included in nuclear incubations, this value increases to about ten times the figure seen in liposomal membranes.

DISCUSSION

Endogenous ∝-TH levels in isolated rat liver nuclei were determined to be 0.045 mol percent. Calculations estimate that this level correlates with a ratio of one molecule of ∝-TH to 967 PUFA moieties in the nuclear membrane. We were unable to find other values in the literature for nuclear levels of ∝-TH in isolated rat liver nuclei. However, studies conducted with other rat liver subcellular organelles have reported values of 3313 and 2100 for the ratio of PUFA moieties to ∝-TH in microsomes (20) and mitochondria (21) respectively.

The present experiment indicates that isolated rat liver nuclei incorporate exogenous ∝-TH, but that the percent of the initial ∝-TH incorporated in nuclei after washing is relatively low. Once taken up by membranes the exogenous ∝-TH effectively inhibited NADPH induced lipid peroxidation. Analogous studies assessing the ability of exogenous ∝-TH to protect rat liver microsomes were performed by Cadenas et al. (22) and Ohki et al. (3). Ohki et al. (3) demonstrated that exogenous ∝-TH inhibited lipid peroxidation even at the lowest concentration incorporated in washed microsomes.

In studies conducted by Cadenas et al. (22), virtually all added ∝-TH remained associated with microsomes following centrifugation. The exogenous ∝-TH inhibited lipid peroxidation but did so fifty times less

effectively than endogenous α-TH. These researchers concluded that α-TH must be present at certain specific sites if it is to efficiently protect membranes against peroxidation. It is important to note that the microsomes supplemented with α-TH in this study were not washed prior to exposure to the peroxidation system. Unlike the preparations utilized in the present study and by Ohki et al. (3). Washing may remove α-TH which is not fully incorporated or bound in the membrane and is therefore less effective in inhibiting lipid peroxidation. Support for this hypothesis is provided by experiments utilizing a model peroxidation system consisting of microsomal structural proteins in lipid micelles (1). Data from this research suggest that vitamin E bound to structural proteins inhibited lipid peroxidation while unbound vitamin E did not.

The present study supports the validity of the α-TH threshold effect in predicting the ability of α-TH to inhibit lipid peroxidation. Membrane α-TH levels above about 0.085 mol percent inhibited nuclear lipid peroxidation, but values below this level were associated with high amounts of peroxidation. The reason for this threshold effect are unknown but may relate to the autocatalytic nature of lipid peroxidation. The initiation of lipid peroxidation starts a free radical chain reaction producing reactive chemical species such as lipid hydroperoxides which can then initiate further lipid peroxi-

dation. The length of these chain reactions ultimately determines how many lipid peroxidation chain branching initiation sites are produced (23). ∝-TH inhibits lipid peroxidation by terminating free radical chain reactions. If enough ∝-TH is present in the membrane, the chain reaction is rapidly terminated and few chain branching initiation sites are produced. In addition, little ∝-TH is oxidized under these circumstances. If however, ∝-TH levels are not sufficient to quickly terminate these chain reactions, large amounts of chain branching initiation sites are produced which subsequently consume existing ∝-TH supplies and peroxidize PUFAs. The threshold effect for ∝-tocopherol has been shown to occur in experiments utilizing liposomes (5, 6) but the present study is the first demonstration that the threshold effect also occurs in native membrane systems.

The addition of GSH to incubations markedly lowered the amount of ∝-TH required to inhibit NADPH induced lipid peroxidation. ∝-TH threshold levels decreased from 0.085 mol percent to 0.040 mol percent following the addition of 1 mM GSH. Previous studies in this laboratory have shown that a nuclear protein is responsible for the GSH dependent inhibition of nuclear peroxidation (10). Two mechanisms may account for the observed synergism between ∝-TH and GSH in the inhibition of lipid peroxidation. The enzymes glutathione transferase (24) and glutathione peroxidase (25) catalyze the GSH dependent

reduction of lipid hydroperoxides to lipid alcohols. Studies indicate that these enzyme activities can be associated with cellular membranes (26, 27). Since lipid hydroperoxides can initiate lipid peroxidation, the reduction of these compounds can contribute to the inhibition of peroxidation. The reduction of lipid hydroperoxides should decrease the number of chain branching initiation sites and thereby increase the effectiveness of existing levels of α-TH. Shlyapnikov and Miller (28), utilizing kinetic models, predicted that agents which reduce lipid hydroperoxides act synergistically with antioxidants such as α-TH to inhibit lipid peroxidation.

Another mechanism which may account for the observed synergism between α-TH and GSH was proposed by Reddy et al. (9). They reported that the GSH dependent inhibition of microsomal lipid peroxidation occurred in microsomal preparations from control rats but not in microsomes prepared from vitamin E deficient rats. On the basis of these data, the existence of a microsomal protein which utilizes GSH to regenerate vitamin E was postulated.

In the nuclear peroxidation system, the addition of GSH reduced the threshold levels of α-TH required to protect against peroxidation. If GSH does regenerate α-TH levels through the actions of a nuclear protein, the rate of lipid peroxidation and α-TH consumption may be reduced but the absolute amount of α-TH required for

protection should remain unchanged. Although the data presented in this report do not discount either hypothesis, the evidence suggests that GSH functions independently of α-TH to inhibit lipid peroxidation. Similar conclusions were reached by Hill and Burk (29) with rat liver microsomes containing various amounts of α-TH. In these experiments, GSH protected against peroxidation even in microsomes containing no detectable α-TH levels. When α-TH was present, GSH extended the effectiveness of the α-TH dependent microsomal protection.

The levels of α-TH needed to protect against peroxidation in nuclear membranes were about five times less than those needed to inhibit peroxidation in liposomal membranes. The increased susceptibility of liposomal membranes as opposed to native membranes was also noted by Fukuzawa et al. (2). The explanation of this effect may relate to differences in membrane structure. Biological membranes contain such compounds as cholesterol which has been shown to suppress lipid peroxidation in liposomal membranes (30). Although cholesterol itself may be oxidized (31), research indicates that cholesterol is more resistant than PUFAs to oxidation (31). Also, other membrane components such as proteins or other antioxidants may protect against lipid peroxidation.

In the presence of 1 mM GSH, one molecule of α-TH protected 1,088 PUFA moieties, while in the absence of GSH

∝-TH protected only 500 PUFA moieties. It is interesting to note that a similar threshold value of ∝-TH was reported by Bieri and Poukka (32). In their experiments, a value of one molecule of ∝-TH to 1,100 PUFA moieties in rat erythrocytes was required to inhibit red cell hemolysis. Endogenous levels of ∝-TH in rat liver nuclei were slightly higher than the threshold levels of ∝-TH needed to protect nuclei in the presence of 1 mM GSH but below threshold values determined in the absence of GSH. Since the concentration of GSH utilized in this study are similar to those found intracellularly (33), this report suggests that GSH may be important in extending the ability of ∝-TH to inhibit lipid peroxidation in vivo.

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THE STATUS OF GLUTATHIONE IN RAT KIDNEY NUCLEI

ABSTRACT

Rat kidney nuclei were isolated by techniques designed to limit the loss of water soluble metabolites. Lyophilized rat kidney powder was disrupted with a Tekmar Tissumizer, and nuclei were purified by discontinuous gradient centrifugation through a glycerol-metrizamide solution at 4°C. Purified nuclei exhibited similar DNA to protein ratios as reported for other rat nuclei isolated by nonaqueous isolation techniques. Results from this study suggest that the concentration of glutathione in the kidney nucleus is similar to that in the cytoplasm under normal glutathione levels. Disparities were detected between the concentration of glutathione found in the nucleus and the cytoplasm following administration of buthionine sulfoximine.

INTRODUCTION

The unique structural properties of the nuclear membrane system make difficult any assessment of the content of small water soluble metabolites in the nucleus. The nuclear membrane system contains numerous sites at which the outer and inner nuclear membranes are joined. These sites are referred to as nuclear pore complexes. The frequency of these nuclear pores within the nuclear membrane varies according to the cell type examined. However, research indicates that the ultrastructure of nuclear pore complexes is remarkably constant in a wide variety of eukaryotic cell types (1). Recent studies have determined that the functional pore radius in isolated rat liver nuclei is 56-59 angstroms (2). The large size of these pores should allow the free diffusion of small water soluble metabolites between the nucleus and cytoplasm. Several reports utilizing both whole cells and isolated nuclei have confirmed the semiporous nature of the nuclear envelope (3-5). In one such study, fluorescent probes attached to various exogenous proteins were injected into the cytoplasm of intact mouse liver cells (3). After a certain period of time, cells were fixed and nuclear fluorescence levels were determined. Results from this study demonstrate that proteins with molecular weights of up to 21,500 can diffuse freely from the cytoplasm to the nucleus in the intact cell.

Despite the presence of these nuclear pores, the concentration of glutathione (GSH) in the nucleus could be different from that of the cytosol if certain physiologic conditions exist. A nuclear membrane transport mechanism for the uptake of GSH could conceivably produce such a nuclear pool of GSH. In a similar manner, the confinement of GSH synthesizing or metabolizing enzymes within the nucleus may produce disparities between nuclear and cytoplasmic GSH levels under certain metabolic conditions. The condition most likely to produce differences in cytoplasmic and nuclear GSH levels involves the relative concentration of nondiffusable GSH binding macromolecules in the cytoplasm and nucleus. This noncovalent binding could involve both proteins and DNA.

The following study utilizes nonaqueous isolation techniques to minimize the loss of nuclear GSH during isolation of rat kidney nuclei. These techniques were used to evaluate nuclear GSH levels and assess the relative concentration of GSH in the nucleus and cytoplasm.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats (Simonsen Labs., Gilroy, CA) weighing 200-300 g were used throughout the course of these experiments. D,L-buthionine sulfoximine (BSO) was dissolved in phosphate buffered saline and injected i.p. at a dose of 300 mg/kg body weight in a total volume of 4 ml/kg body weight. Rats were killed six hours after the injection of BSO.

Chemicals

D,L-buthionine sulfoximine and metrizamide were purchased from Sigma Chemical Co. (St. Louis, MO). Anhydrous glycerol was prepared by heating glycerol to 160°C with stirring followed by storage over P_2O_5.

Nonagueous Isolation of Kidney Nuclei

Rat kidney nuclei were prepared according to the procedures of Kennedy and Mathias (6) with modifications. Rats were anesthetized with ether then killed by decapitation. Kidneys were immediately removed and then frozen in isopentane chilled to -160°C in liquid nitrogen. Kidneys were then crushed in a mortar and pestle chilled with liquid nitrogen, and the resulting frozen kidney powder was lyophilized for 48 hours. Following lyophilization, the kidney powder was crushed to a fine powder under a stream of argon in a mortar and pestle chilled in a bath of dry ice and acetone. The tissue powder was

stored in a desiccator containing P_2O_5 and allowed to warm to room temperature. Whole cell determinations were performed utilizing this kidney powder.

Isolated kidney nuclei were prepared by suspending the lyophilized kidney powder in a solution of 2.0 percent metrizamide (w/v) dissolved in anhydrous glycerol at 4°C. Once suspended, the tissue suspension was homogenized for a total of three minutes with a Tekmar Tissumizer. Homogenization was achieved with a series of short pulses to maintain the temperature of the homogenate at or below 4°C. The homogenate was then layered over 1 ml of 2.0 percent metrizamide-glycerol in a series of ultracentrifuge tubes and centrifuged at 30,000 RPM for one hour at 4°C using a Beckman SW 40 rotor. After centrifugation, tubes were drained of their contents and the sides of the tubes were wiped clean with 95 percent ethanol. Microscopic examination revealed less than 1 percent whole cell contamination in nuclear pellets prepared by these procedures.

Biochemical Determinations

Both nuclear pellets and whole cell kidney powder were suspended in 10 percent perchloric acid and centrifuged at 15,000 x g for two minutes. The pellets formed were assayed for DNA and protein, while supernatants were utilized for GSH and aspartate determinations. DNA was measured according to the procedures of Erwin et al. (7). Protein levels were

determined according to the procedures of Lowry et al. (8) with modifications described by Peterson (9). Aspartate and GSH levels were quantified by the methods of Reed et al. (10) as modified by Fariss and coworkers (11).

RESULTS AND DISCUSSION

The nonaqueous nuclear isolation procedures utilized in this study consist of three distinct steps. First the tissue is removed, frozen and lyophilized to remove intracellular water. The lyophilized tissue is then homogenized in cold anhydrous glycerol to free the nucleus from the cell. Previous studies have demonstrated that the solubility and diffusion of small water soluble metabolites is negligible in cold glycerol (12). Once the tissue is homogenized, nuclei can be separated from other cellular constituents by density gradient centrifugation. Since the disruption of cells is usually incomplete, adequate purification of nuclei only occurs if whole cells and nuclei have different densities. Previous studies have failed to detect such a density difference in rat liver tissue. However, in this study, rat kidney nuclei could be separated from whole cells by centrifuging homogenates through a glycerol-metrizamide solution.

The physical characteristics of kidney nuclei purified by nonaqueous isolation techniques are shown in Table V-1. The DNA content of these nuclei are in close agreement with other published values (6,13), and the ratio of protein to DNA is also similar to those obtained for rat liver nuclei isolated in glycerol (6).

The GSH content of rat kidney nuclei and whole cells is listed in Table V-2. In control experiments, nuclear

TABLE V-1

DNA and Protein Content of Isolated Kidney Nuclei Purified by Nonaqueous Isolation Techniques

	DNA ($ug/10^6$ nuclei)	Protein ($ug/10^6$ nuclei)	Protein/DNA
Nuclei	9.3 ± 0.7 (5)[a]	89.7 ± 1.7 (3)	9.6
Whole Cells	-	-	95.9

Purification Factor[b]	10
Yield[c]	10%

a Mean Values ± S. E.
Number of observations in parentheses.

b Based on ratio of protein/DNA in whole cells and isolated nuclei.

c Yield based on percent DNA recovered in isolated nuclei from total DNA content of homogenate.

TABLE V-2

Glutathione Content of Kidney Cells and Isolated Kidney Nuclei Purified by Nonaqueous Isolation Techniques

Nuclear GSH

Treatment	nmol GSH/ 10^6 nuclei	nmol Aspartate/ nmol GSH	Percent of Whole Cells
Control	0.48 ± 0.08 (8)[a]	1.7	4.9
+ BSO[b]	0.03 ± 0.01 (5)	13.2	0.9

Whole Cell GSH

Treatment	nmol GSH/ 10^6 nuclei[c]	nmol Aspartate/ nmoles GSH
Control	9.84 ± 2.83 (8)	1.0
+ BSO[b]	3.43 ± 0.60 (6)	2.2

a Mean values ± S. E.
Number of observations in parentheses.

b BSO treatments as described in experimental procedures section.

c Values determined by measuring the DNA content of tissue powder and assuming 9.3 ug DNA per 10^6 cells.

GSH levels accounted for about 5 percent of the total whole cell values of GSH. To determine whether the concentration of GSH in the nucleus is the same as in the cytosol, the volume of the nucleus must first be known. Since the kidney is composed of several cell types, this value is at best approximate. Microscopic examination of kidney cells suggests that the average volume of kidney nuclei is about 5 percent of the whole cell (data not shown).

The ratio of aspartate to GSH in whole cell and nuclear preparations were also determined. Aspartate is a small water soluble metabolite with similar charge properties as GSH, and as such should have analogous diffusion and ionic binding properties. A comparison of the ratio of aspartate to GSH reveal only slight differences between these values in whole cells and the nucleus. On the basis of these data, it appears that GSH concentrations in the nucleus and cytosol are similar in kidney cells and can be accounted for by the free diffusion of GSH between the cytosol and nucleus.

Considerably different levels of GSH were detected when rats were pretreated with BSO. BSO has been shown to inhibit GSH synthesis and to deplete GSH in variety of organs (14). In this experiment, administration of BSO reduced whole cell kidney GSH levels to about 34.9 percent of controls after six hours. Nuclear GSH levels were depleted to a much greater extent (about 6 percent of

control nuclei) than that of whole cells.

This pattern of GSH depletion was also reflected in the ratio of aspartate to GSH. Measurements determined that the aspartate to GSH ratio was 13.2 in the nucleus but only 2.2 in whole kidney cells after BSO administration. Thus, aspartate levels were not depleted in the nucleus to the same extent as GSH. Since aspartate and GSH would be expected to have analogous binding and diffusion properties, these results most likely cannot be explained by the loss of GSH during the isolation of nuclei.

In vitro studies conducted by Sugiyama and Kaplowitz (15) predict that under normal physiological conditions 25 to 30 percent of all rat liver cytosolic GSH is bound to high affinity binding sites on cytosolic proteins. The remainder of the cytosolic GSH remains free or unbound to macromolecules. Progressively higher percentages of GSH were bound to proteins when lower GSH concentrations were incubated with rat liver cytosolic proteins.

These results may explain why nuclear GSH is depleted to a much greater extent than cytosolic GSH in the kidney following BSO administration. Although the nuclear membrane is semiporous and should not prevent the diffusion of free GSH between the nucleus and cytosol, it may prevent the interchange of high affinity binding proteins between the nucleus and cytosol. In this model, the high affinity binding sites are saturated under normal

physiologic conditions and free GSH diffuses evenly throughout the cytosol and nucleus. However, when GSH levels are reduced, the relative contribution of the free GSH to the overall cellular levels of GSH increases. If the quantity of GSH binding proteins is higher in the cytosol than the nucleus, cytosolic concentrations of GSH may be considerably greater than those found in the nucleus. Further research is required to test this hypothesis.

The present study indicates that under normal physiologic conditions GSH is found in the nucleus of rat kidney at concentrations similar to those found in the cytoplasm. The results also suggest that the depletion of GSH may produce significant differences between nuclear and cytosolic GSH concentrations.

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CONCLUSION

The results from this study indicate that isolated nuclei are susceptible to lipid peroxidation. Nuclear peroxidation could be initiated by the addition of either NADPH or ascorbate in conjunction with chelated ferric iron. The addition of GSH protected isolated nuclei against lipid peroxidation by inducing a lag period prior to the onset of lipid peroxidation. The duration of this GSH induced lag period was directly related to the concentration of GSH present in the incubation mixture. Higher concentrations of GSH produced longer lag periods. This glutathione dependent protection could be abolished by preincubating isolated nuclei with disulfides, thiol modifying reagents or by treating nuclei with heat or trypsin. These same treatments also inhibited the GSH dependent reduction of cumene hydroperoxide. On the basis of these results, it was concluded that a protein containing an essential sulfhydryl group and exhibiting glutathione peroxidase activity is responsible for the glutathione dependent inhibition of lipid peroxidation. Experiments suggest that this protein is a glutathione transferase associated with the nuclear membrane.

Partially purified nuclear transferase fractions catalyzed the GSH dependent reduction of cumene hydroperoxide, tert-butyl hydroperoxide, and lipid hydroperoxides. It is proposed that this nuclear glutathione

transferase inhibits lipid peroxidation by reducing lipid hydroperoxides.

Vitamin E also protects isolated nuclei against lipid peroxidation. Research indicates that vitamin E incorporated into nuclei above a certain critical level inhibits nuclear lipid peroxidation. When vitamin E levels were below this value, vitamin E was rapidly consumed and lipid peroxidation was not inhibited. The addition of GSH to nuclear incubations reduced the levels of vitamin E required to inhibit lipid peroxidation. The data suggest that this observed synergism is not due to the regeneration of vitamin E by GSH but rather occurs as a result of the independent actions of vitamin E and GSH.

Several studies report that small water soluble metabolites diffuse freely from the nucleus to the cytosol. Isolation of rat kidney nuclei by techniques designed to limit the diffusion of these metabolites indicate that the concentration of GSH is similar in the nucleus and the cytosol. However, administration of the GSH depleting agent BSO to rats, produced disparities between the concentration of GSH in the nucleus and the cytosol. GSH was depleted to a much greater extent in the nucleus than in the cytosol. These results imply that under certain metabolic conditions the concentration of GSH in the nucleus may be less than that in the cytosol, and that the nucleus may be at greater risk than the cytoplasm to reactions involving detoxification with GSH.

In conclusion, GSH appears to play a significant role in the protection of cell nucleus against lipid peroxidation.

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