Dietary choline restriction causes complex I dysfunction and increased $\text{H}_2\text{O}_2$ generation in liver mitochondria

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Removal of choline from the diet results in accumulation of triglycerides in the liver, and chronic dietary deficiency produces a non-genotoxic model of hepatocellular carcinoma. An early event in choline deficiency is the appearance of oxidized lipid, DNA and protein, suggesting that increased oxidative stress may facilitate neoplasia in the choline deficient liver. In this study, we find that mitochondria isolated from rats fed a choline-deficient, l-amino acid defined diet (CDAA) demonstrate impaired respiratory function, particularly in regard to complex I-linked (NADH-dependent) respiration. This impairment in mitochondrial phosphatidylcholine. Moreover, hydrogen peroxide ($\text{H}_2\text{O}_2$) generation is significantly increased in mitochondria isolated from CDAA rats compared with mitochondrial from normal rats, and the NADH-specific yield of $\text{H}_2\text{O}_2$ is increased by at least 2.5-fold. These findings suggest an explanation for the rapid onset of oxidative stress and energy compromise in the choline deficiency model of hepatocellular carcinoma and indicate that dietary choline withdrawal may be a useful paradigm for the study of mitochondrial pathophysiology in carcinogenesis.

Introduction
Dietary choline deficiency produces a non-genotoxic model of liver cancer in rats (1,2). Choline restriction produces a well-defined pattern of temporal alterations in the liver while leaving other organs largely unaffected (3). Within 24 h of choline restriction, triglycerides begin to accumulate in rat liver with all regions of the liver being affected within 4–5 days (3). Evidence for membrane peroxidation, specifically the formation of conjugated dienes in nuclear and mitochondrial membranes, has been shown within 1–5 days (4–6) and persists throughout choline restriction-induced neoplasia (7). Oxidative damage to DNA, particularly the formation of 8-oxodeoxyguanosine lesions, occurs within the time frame of lipid peroxidation (7). Some hepatocyte death occurs beginning at 5–14 days (8), and by 10 weeks preneoplastic nodules are clearly evident (7,9). True hepatocellular carcinomas are present after one year of prolonged choline deficiency (2).

The early appearance of oxidative lesions in choline deficient liver and the observation that antioxidants can inhibit carcinogenesis induced by choline withdrawal (7,9,10), combined with the known ability of oxidants to induce carcinogenesis (11), suggests that choline deficiency either causes increased generation of oxidants in liver or else inhibits the liver's ability to detoxify oxidant species. No mechanisms have been proposed to support either alternative. Reasoning that mitochondria are a major source of reactive oxygen species (ROS) in liver (12), and considering that phospholipid composition is altered significantly in choline deficient mitochondria (13–15), we hypothesized that mitochondrial respiration might be altered during choline withdrawal so as to produce an increased leakage of ROS which might predispose the liver to neoplasia. Here, we report that mitochondria isolated from rats fed a choline deficient, l-amino acid-defined diet (CDAA) for 3–7 days are significantly altered with respect to rats fed a choline sufficient, l-amino acid-defined (CSAA) diet and with respect to animals fed a basal diet of normal rat chow. Complex I function (NADH dehydrogenase activity or NADH-linked respiration) is significantly reduced in CDAA compared with normal mitochondria. The ability to detoxify oxidant species. No mechanisms have been proposed to support either alternative. Reasoning that mitochondria are a major source of reactive oxygen species (ROS) in liver (12), and considering that phospholipid composition is altered significantly in choline deficient mitochondria (13–15), we hypothesized that mitochondrial respiration might be altered during choline withdrawal so as to produce an increased leakage of ROS which might predispose the liver to neoplasia. Here, we report that mitochondria isolated from rats fed a choline deficient, l-amino acid-defined diet (CDAA) for 3–7 days are significantly altered with respect to rats fed a choline sufficient, l-amino acid-defined (CSAA) diet and with respect to animals fed a basal diet of normal rat chow. Complex I function (NADH dehydrogenase activity or NADH-linked respiration) is significantly reduced in CDAA compared with normal mitochondria.

Materials and methods
Reagents
Fluorogenic substrates and standards were purchased from Molecular Probes (Eugene, OR). NADH and succinate were purchased from Sigma (St Louis, MO). All other reagents were of the highest available purity.

Animals and diet
Male Wistar rats, 100–200 g, were purchased from Charles River Laboratories (Wilmington, MA) and maintained in the Oklahoma Medical Research Foundation Laboratory Animal Care Facility until use. In each experiment, animals were divided into two groups of five animals each and fed either a basal diet (normal chow) or a CDAA diet as previously described (16), or a CSAA diet which was identical in every respect to the CDAA diet except that choline was not omitted from the formulation. Separate experiments were conducted with 1, 3 and 7 days of CDAA administration. The basal diet was Purina 5001 (Ralston Purina, St Louis, MO). The CDAA and CSAA diets were purchased from Dyets (Bethlehem, PA). Animals were weighed daily.

Abbreviations: CDAA, choline-deficient l-amino acid defined; CSAA, choline-sufficient l-amino acid defined diet; MS, mass spectrometry; NADH, nicotinamide adenine dinucleotide (reduced); PC, phosphatidyl choline.
No significant differences were seen in weight gain between CDDA and basal diets.

Isolation of liver mitochondria

Mitochondria were isolated similar to previously described methods (12,17,18,21–26). Approximately 3 g slices of liver were immersed in ~25 ml ice-cold isolation medium [0.3 M sucrose, 25 mM tris(hydroxymethyl) aminomethane, 2 mM EDTA, pH 7.3] and finely minced by brief disruption (2–5 s) with a Polytron (Brinkman Instruments, Westbury, NY) motor-driven tissue homogenizer followed by two strokes of a motor-driven glass walled dounce-type homogenizer equipped with a teflon pestle (0.25 mm clearance). Tissue thus homogenized was centrifuged at 10–15°C and 500 g for 15 min in a fixed-angle rotor. Supernatant was decanted and centrifuged at 9000 g for 15 min. The pellet from the second centrifugation was gently dispersed into 20 ml isolation medium and recentrifuged at 10,000 g for 15 min, and this wash was repeated once more. The final pellet was resuspended in 4.5 ml isolation medium and rotated for 10–20 min at 60 r.p.m. in order to gently disperse particulates. Protein content was determined by the method of Lowry (19) and diluted to 4 mg/ml by addition of isolation medium.

Analysis of liver and mitochondrial protein

Total protein from liver, or protein from isolated mitochondria, was electrophoresed on 12% polyacrylamide gels in the presence or absence of 1 mM dithiothreitol and visualized by staining with Coomassie blue. For measurement of protein carbonyl content (an index of protein oxidation), western blot analysis was performed after dinitrophenyl hydrazine derivatization using a commercially available assay (Oxyblot™, Intergen, Purchase, NY) performed according to the manufacturer’s instructions. Blots were developed using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK) and digitally quantified. As an index of liver damage, plasma aspartate aminotransferase (AST) activity was measured. Plasma was separated from whole blood withdrawn from the femoral artery of specific rats which were sacrificed for histopathology at indicated time periods after initiation of the CDDA diet. AST activity was determined by an established assay (27).

Tandem mass spectrometry of phosphatidylcholines

Lipid was extracted from mitochondria using a mixture of 3:1 chloroform:methanol and analyzed for PC distribution by a variation of an established method (20). An aliquot (1 ml) of mitochondrial preparation (4 mg/ml) was mixed with 1 ml chloroform–methanol mixture in a glass vial and shaken vigorously. The chloroform layer was isolated and used for mass spectrometry without further treatment. Mass spectra were obtained on a VG Quattro triple stage mass spectrometer (VG Biotech, Altrincham, UK) using a fast-ion bombardment (FIB) probe head with a potassium cation beam. Ethanolamine was used as the liquid matrix for FIB ionization. Approximately 5 µl of the lipid chloroform extract was evaporated from the liquid matrix at ambient temperature. Ethanolamine (2 µl) was then added to obtain a homogeneous solution of lipid. A drop of the ethanolamine solution was applied to the tip of the FIB sampler and inserted into the spectrometer to initiate the measurement. Spectra were obtained in the parent-search mode with the third stage detector fixed to the phosphocholine fragment (m/z = 184) while the first stage detector was swept to search for molecular ions that were precisely mass matched to the identification of the peak positions (m/z) was confirmed using a 1:1 mixture of dipalmyr-10yl phosphatidylcholine and dimiristoyl phosphatidylcholine.

Determination of mitochondrial O2 consumption

State 4 oxygen utilization by mitochondrial preparations was followed using a stirred, Clark-type oxygen electrode maintained at 30°C. State 4 oxygen utilization by mitochondrial preparations was followed using a stirred, Clark-type oxygen electrode maintained at 30°C. The electrode system was calibrated by addition of 5 mM potassium phosphate, 80 mM KCl pH 7.3) and 1 mM substrate in a vacuum desicator at ambient temperature for 6 h. NADH oxidation assays were then performed as described above. The electrode system was calibrated by addition of 5 mM potassium phosphate, 80 mM KCl pH 7.3) and 1 mM substrate in a vacuum desicator at ambient temperature for 6 h. NADH oxidation assays were then performed as described above.

Measurement of hydrogen peroxide generation

Hydrogen peroxide produced by stimulated mitochondria (state 4 conditions) was measured fluorometrically using a horseradish peroxidase (HRP)-dependent dichlorofluorescein (DCF) assay similar to previously described methods (12,17,18,21). Reaction mixtures were prepared in 96-well clear microplates (Fischer Scientific, St Louis, MO). Reaction mixtures contained 10 µl HRP (initially at 2 mg/ml), 2 µl reduced dichlorofluorescein diacetate (H2DCFDA, predissolved to 10 mM in absolute ethanol), 124 µl KHP and 40 µl mitochondria (initially at 4 mg/ml in isolation medium). Reactions were initiated by addition of 20 µl concentrated substrate (NADH or succinate in KHP). H2O2 production was measured as pmol H2DCFDA oxidized/mg protein/min using a Molecular Devices Fmax™ instrument (Sunnyvale, CA).

Fig. 1. Mass distribution of phosphatidyl choline (PC) in liver mitochondria from rats fed CDDA or basal diets. The mass spectrophotograph shows a typical PC distribution after 7 days of CDDA administration. Note the relative increase in higher molecular weight PC at 3 and 7 days of dietary restriction (table, inset; data represent mean ± SD of five animals per treatment group per time point; * indicates P < 0.01, Student’s t-test). Operating at 37°C with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Authentic dichlorofluorescein was used as a standard. In specific experiments, rotenone was added in 1 µl volumes after dissolution in absolute ethanol. Specific experiments were conducted to confirm that mitochondrial stimulants and inhibitors used in this study did not affect HRP activity or HRP-catalyzed, H2O2-dependent H2DCFDA oxidation. Substitution of the fluorogenic esterase substrate calcein for H2DCFDA indicated that total esterase activity of mitochondrial preparations did not differ between normal and CDDA treatment groups.

Measurement of complex I NADH-dehydrogenase activity

To measure complex I-dependent NADH oxidation, 40 µl mitochondria (4 mg/ml) were mixed with 140 µl KHP and 20 µl NADH (at 0–1 mM final concentration) in 96-well clear microplates. NADH oxidation was monitored spectrophotometrically at 340 nm and 37°C using a ThermoMax™ kinetic microplate reader ( Molecular Devices, Sunnyvale, CA). In specific experiments, 10 µl ubiquinone (Q10) was also included in the reaction mixture. Rotenone-sensitive (e.g. complex I-specific) and rotenone-insensitive components of NADH oxidation were determined by addition of excess rotenone (final concentration 10 µM) to the reaction mixture (12). Rotenone was found to inhibit approximately 90% of the NADH dehydrogenase activity in normal mitochondrial preparations.

Extraction of mitochondrial lipids from mitochondrial protein

Mitochondrial lipids were extracted in ace tone containing 10% vol/vol water, according to established methods (22,23). Mitochondria (at 4 mg/ml protein) were mixed with 90% ace tone/10% water in a ratio of 1 ml mitochondrial preparation to 5 ml ace tone/water mixture. After gentle mixing for 10 min, samples were centrifuged at 5000 g and supernatants (lipid fraction) poured off. Pellets were redispersed in 1 ml ace tone/water mixture. One half of each resuspended pellet (protein) fraction was reconstituted with one half of each supernatant (lipid) fraction. Ace tone was then removed by placing all samples in a vacuum desicator at ambient temperature for 6 h. NADH oxidation assays were then performed as described above.

Results

Time course of lipid changes in liver mitochondria

Tandem mass spectrometric analysis performed on extracted mitochondrial lipids indicated that PC pools were substantially altered within 3 days after initiating CDDA, and this perturbation was maintained to 7 days, the longest time period studied (Figure 1). The mass spectrum of lipids extracted from rats on the basal and CDDA diets were essentially identical. Essentially no alteration in PC pools were observed after only 1 day of choline restriction. Because the mass spectra were recorded in the so-called parent search mode, the molecular
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Fig. 2. Effect of CDAA diet on mitochondrial respiration. Oxygen consumption by state 4 liver mitochondria was monitored using an O₂ electrode, and respiratory ratio was calculated as the rate of O₂ consumption after addition of substrate (1 mM succinate or 1 mM NADH) divided by the basal rate of O₂ reduction before substrate addition. Rates of O₂ consumption in normal mitochondria were 10–15 nmol/mg/min under succinate stimulation. Data indicate mean ± SEM; *, P < 0.05; **, P < 0.01 by Student’s t-test for paired samples; n, five pairs of animals.

Fig. 3. Effects of CDAA diet on complex I function. Liver mitochondria from rats were isolated after 7 days on the CDAA or basal diet. (A) NADH dehydrogenase activity of liver mitochondria. (B) Rotenone sensitivity of CDAA or normal mitochondria indicates similar sensitivity to the complex I-specific inhibitor (half-maximal inhibitory concentration approximately 7 nM, both groups). (C) Lineweaver–Burke analysis indicating non-competitive nature of complex I inhibition in CDAA liver mitochondria. The mitochondrial preparations in (C) were lysed by freezing at −80°C and thawing. Data indicate mean ± SEM; n, five pairs of animals for each treatment and group.

effect was maintained if the mitochondria were sonicated or frozen and thawed, though both treatments increased NADH oxidation rate. Addition of ubiquinone to CDAA mitochondria did not restore NADH oxidation capacity (data not shown). Addition of rotenone to both normal and CDAA mitochondria caused a similar dose–response of complex I inhibition, with IC₅₀ values of approximately 7 nM in both cases (Figure 3B). Lineweaver–Burke analysis indicated that the inhibition of NADH oxidation induced by choline deficiency was uncompetitive with NADH (Figure 3C). These data indicate that the effect of CDAA on complex I probably occurs downstream from the rotenone-binding site, and does not reflect loss of NADH access to the NADH oxidation site.

Complex I dysfunction is associated with increased H₂O₂ synthesis

Mitochondria treated with the complex I inhibitor rotenone, or other downstream inhibitors of electron transport, generate increased amounts of H₂O₂ during NADH-stimulated state 4 respiration (18). Accordingly, it might be expected that liver mitochondria from CDAA rats would generate more H₂O₂ per unit NADH than would normal mitochondria. As illustrated in Figure 4, the NADH-dependent H₂O₂ generation rate of CDAA mitochondria is approximately 35% greater than the corresponding rate in normal mitochondria while succinate-stimulated H₂O₂ flux is increased by 7.5% (not statistically significant) (Figure 4A). The yield of H₂O₂ in normal liver mitochondria was approximately 2% of the NADH consumed, very near to previously reported estimates of mitochondrial
Lipids from CDAA mitochondria inhibit complex I function in protein extracted from normal mitochondria. Rats were fed a CDAA or basal diet for 7 days, liver mitochondria were isolated and lipids extracted as described in experimental procedures. Lipids and proteins were reconstituted as indicated. Note that NADH dehydrogenase activity of CDAA protein reconstituted with CDAA lipid remains diminished relative to the activity in normal protein reconstituted with CDAA lipid. Reconstitution of normal protein with CDAA lipids inhibits NADH dehydrogenase function in the normal protein fraction. Contrastingly, normal lipid reconstituted with CDAA protein fails to restore NADH dehydrogenase activity of the CDAA protein extract. Data indicate mean ± SEM; *, $P < 0.05$ by Student’s $t$-test; $n$, five animals/group.

Fig. 4. Effects of CDAA diet on H$_2$O$_2$ biosynthesis in liver mitochondria. Liver mitochondria were isolated after 7 days on the CDAA or basal diet, unless otherwise indicated. (A) H$_2$O$_2$ production by liver mitochondria during stimulation with 1 mM NADH or 1 mM succinate. (B) NADH-dependent H$_2$O$_2$ yield expressed relative to NADH consumption (mitochondria were stimulated with 1 mM NADH). (C) Time course of mitochondrial dysfunction during choline restriction. Data indicate mean ± SEM; *, $P < 0.05$; **, $P < 0.01$ by Student’s $t$-test; $n$, five animals/group at each time point.

H$_2$O$_2$ leakage (12). When the H$_2$O$_2$ production rate was normalized to the NADH oxidation rate, H$_2$O$_2$ yield in CDAA mitochondria was found to increase at least 2.5-fold after 7 days of CDAA diet (Figure 4B). When NADH oxidation rates and NADH-dependent H$_2$O$_2$ generation are compared at different durations of choline restriction, a consistent temporal pattern is observed (Figure 4C). Very little alteration is seen in CDAA mitochondria after 1 day of CDAA diet, while significant dysfunction is seen at 3 and 7 days. NADH utilization was very similar in mitochondria isolated from rats fed the normal diet and the CSAA diet (Figure 4C). In all cases, decreases in NADH oxidation rate within the CDAA group of animals were correlated with increases in H$_2$O$_2$ flux. The time course of complex I dysfunction therefore closely parallels the time course of alteration of PC chains (Figure 1).

Lipid dependence of complex I dysfunction in CDAA livers

It is possible to extract >80% of total PC from mitochondria, and to reconstitute lipids and protein from different mitochondrial isolates (23–24). Since a correlation was found between PC chain length alterations and complex I function, an experiment was undertaken to recombine lipids from CDAA mitochondria with protein of normal mitochondria. Although the extraction procedure greatly diminished complex I function in normal mitochondria, it was found that reconstitution of normal mitochondrial protein with CDAA lipids produced a significant inhibition of complex I function (Figure 5). In fact, all combinations of protein and lipid displayed complex I inhibi-
mitochondria. Complex I and II-linked respiration are both diminished by choline withdrawal, though the complex I dysfunction is relatively more severe. A 70% decrease in complex I specific (i.e. rotenone-sensitive) NADH oxidation occurs between 1–3 days of choline restriction. Most notably, the complex I dysfunction is accompanied by an increased H$_2$O$_2$ leakage from the mitochondria during complex I-linked respiration. The deficit in NADH utilization is not likely to result from lesions to electron transport elements at or beyond complex III, because inhibition at such sites should suppress complex I and complex II-linked respiration equally and should greatly accelerate succinate-stimulated H$_2$O$_2$ production (18). The onset of mitochondrial dysfunction occurs during a time frame in which increased lipid peroxidation is reported (4–7) and well before the appearance of preneoplastic nodules.

One may question whether the mitochondrial lesions that occur as a result of choline withdrawal actually contribute to hepatocyte death or, alternatively, merely reflect the presence in the liver of dead and dying cells. Several arguments mitigate against the latter hypothesis. First, mitochondria are not likely to survive intact after cell death in a form which can be isolated by the differential centrifugation protocols employed in this investigation. Second, loss of mitochondrial membrane integrity (which might be expected during cell death) increases rather than decreases the rate of NADH-linked oxygen consumption by exposing the NADH binding site of complex I (present on the matrix side of the inner mitochondrial membrane). Lyed mitochondria appear grossly uncoupled during oxygen consumption assays and this was not the case for those isolated from CDAA livers. Moreover, experimental lysis of the mitochondria does not abrogate the between-group differences in mitochondrial respiratory function (Figure 3). Third, any numerical loss of mitochondria which might occur as a result of hepatocyte death would be expected to diminish complex I and II activity uniformly; however, the effects observed in the CDAA model are selectively mapped to complex I (Figure 2). Therefore, the mitochondrial damage elicited by choline withdrawal probably represents a contribut-

Several explanations are possible for the effect of choline restriction on mitochondrial function. The correlation between lipid alterations and complex I dysfunction, and the ability of lipids extracted from CDAA mitochondria to inhibit complex I function when reconstituted into normal mitochondria (Figure 5), argues that the complex I deficit results from changes induced in the lipid membrane by choline deficiency. The fact that normal lipid will not restore NADH dehydrogenase activity to the CDAA protein fraction (Figure 5) may reflect incomplete extraction of inhibitory lipid components from CDAA mitochondria. A lipid influence on mitochondrial function is not without precedence. For example, ceramide can inhibit complex III activity (25) while arachidonic acid inhibits complex I activity (26), though in our hands such effects require relatively high concentrations (>100 µM) of these lipid metabolites. Conceivably, an increase in average length of lipid chains could impart a biophysical effect on electron transfer through complex I. In such a model, an increase in the lipid bilayer thickness might interfere with normal docking of ubiquinone with complex I, thereby inhibiting ubiquinone reduction and forcing electron leakage from reduced centers within complex I and upstream from the ubiquinone docking site. However, we see no inhibition of complex I function during experiments in which saturated PC of increasing chain length (16–20 carbon units) is experimentally added to normal mitochondria (data not shown). Alternatively, unusual lipid metabolites present in CDAA mitochondria might directly and preferentially inactivate complex I. Such a lipid perturbation might also affect complex II-linked respiration, which is altered in CDAA liver mitochondria, albeit to a lesser extent. We have found that complex II function in brain mitochondria is generally more resistant to chemical inhibition, and produces relatively less H$_2$O$_2$ than complex I-linked respiration (18). The finding that complex I-linked respiration is more resilient in choline deficient liver is therefore not anomalous.

Deficiency in mitochondrial respiration might be expected to cause diminution of ATP levels in liver. Such ATP deficits have been reported previously by different groups studying choline and lipotrope deficiencies (28–30), although the dietary paradigms used in these previous investigations may not be directly comparable to the CDAA diet used in the present study. ATP deficits during choline withdrawal have been explained on the basis of diminished purine synthesis due to methyl group deficiency (28); however, in light of the present observations, mitochondrial dysfunction may also be a contributing factor to altered nucleotide concentrations in choline deficient liver.

Altered phospholipid metabolism may be a common feature of cancerous tissue. For instance, phosphocholine concentration reportedly increases 15–20-fold in human tumor cells or rat sarcoma cells compared with non-malignant cells proliferating at a similar rate (31–33) and may be accompanied by a decrease in ATP (34). In another recent study, total phosphatidylcholines were found to be elevated by 20% in the serum of patients with mammary cancer, and moreover, the ratio of longer-chain to shorter-chain PC was significantly increased (35). Such findings have been taken to suggest an association of malignancy with induction of phospholipid synthesis and breakdown (34). The intriguing possibility therefore arises that altered lipid metabolism in certain kinds of cancerous tissue may affect the kinetics of oxidant generation, thereby facilitating
carcinogenesis. This possibility remains to be tested in future investigations.

The present study links mitochondrial complex I dysfunction and concomitant hydrogen peroxide generation with the onset of a known carcinogenic phenomenon. Oxidant species, particularly hydrogen peroxide and derived hydroxyl radicals, are known genotoxic carcinogens (11). The CDAAD diet model of carcinogenesis, however, does not involve any known genotoxic lesions, despite serious efforts to discover such events. It is therefore intriguing to speculate that the $H_2O_2$ generation and energy deprivation which occur in the livers of choline-restricted rats elicits a neoplastic transformation by some means other than direct mutation of proto-oncogenes or tumor suppressor genes. Oxidative inactivation of sensitive thiol-dependent protein and lipid phosphatases as a consequence of increased peroxide flux may predispose hepatocytes to neoplasia in the absence of genotoxic changes. This hypothesis is plausible for three reasons. First, many proto-oncogenes encode either protein kinases which regulate cell cycle progression (e.g. cyclin-dependent kinases), or elements of G protein-coupled signal transduction which operate on protein kinase cascades (36). Disruption of these genes by mutation facilitates neoplasia. Second, recent discoveries have been made of tumor suppressor genes which encode phospholipid phosphatases (i.e. the PTEN enzyme) with cysteine-dependent, tyrosine phosphatase-like catalytic domains (37–39). Mutation of the PTEN phospholipid phosphatase leads to neoplasia (37,38), and full in vitro activity of the enzyme requires high concentrations of thiol reducing agents (39). Third, protein tyrosine phosphatases have been shown to readily scavenge $H_2O_2$ in a self-destructive reaction, forming meta-stable sulfenic acid derivatives at the active site of the enzyme (40). Thus, chronic oxidative inactivation of protein or lipid phosphatases which regulate cell cycle progression might produce a phenotype similar to that observed in cells with correspondingly mutated 16. Nakae,D., Yoshiji,H., Maruyama,H., Kinugasa,T., Denda,A. and Konishi,Y . (1994) Preventive affects of various antioxidants and speci

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