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Increased calcium-independent phospholipase A_2 activity in vitamin E and selenium-deficient rat lung, liver, and spleen cytosol is time-dependent and reversible

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Long-Evans hooded rats maintained on vitamin E and selenium-deficient diets for 6 weeks from weaning exhibited a pattern of enhancement of calcium-independent phospholipase A_2 activity in spleen cytosol similar to that previously reported for the lung and liver. The spleen cytosols from vitamin E and selenium deficient rats had approximately 5 fold higher activity than the samples from animals maintained on diets sufficient in these two nutrients (control) or deficient in either nutrient alone. The calcium-dependent PLA_2 activity was about 6 fold higher in spleen cytosol of vitamin E and selenium deficient rats compared with cytosolic samples from animals on the other three diets. Time course studies indicated that in rats the calcium-independent phospholipase A_2 activity in lung, liver, and spleen increased initially between 4 and 6 weeks of consuming the diets deficient in both vitamin E and selenium, and increased even further again at 7 weeks. This bi-phasic response to the deficiency occurred 1 week after indicators of vitamin E and Se status had reached minimum levels. When animals that were maintained on the deficient diet for 6 weeks consumed the control diet for 1 week the phospholipase A_2 activity of lung, liver, and spleen was not different than the activity of the control animals. The malondialdehyde concentration of lung and spleen measured at 6 and 7 weeks correlated positively with the calcium-independent phospholipase A_2 activity. These results indicate that deficiency of vitamin E and selenium in the rat leads to a bi-phasic increase in calcium-independent PLA_2 activity in rat lung, liver, and spleen, and that the initial increase can be reversed by partial repletion of the two antioxidant nutrients. (J. Nutr. Biochem. 7:366–374, 1996.)

Keywords: phospholipase A_2; selenium; vitamin E; rat; lung; malondialdehyde

Introduction

In aerobic organisms, cellular oxygen undergoing reduction can form reactive oxygen species (ROS), which have the potential to damage cellular macromolecules.[1] The formation of ROS can increase in response to environmental influences such as exposure to oxidants or disease states such as ischemia-reperfusion injury.[2] In healthy cells the level of ROS is kept in balance by a number of cellular defense systems that involve both nutrient antioxidants, such as vitamin E, and mineral-protein enzymes, such as selenium-containing glutathione peroxidase.[3] These systems act by preventing the formation of ROS or by detoxifying the ROS once produced. Cellular defense also includes enzyme systems capable of repairing ROS-induced damage, such as endonucleases, lipases, and proteases.[4] When the balance is disturbed by accelerated ROS formation or impaired defense systems, the oxidation of cellular DNA, protein, and lipid that results can lead to tissue damage and result in a state of oxidative stress. A number of studies implicate tissue damage by ROS as a contributing factor in the devel-

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opment of symptoms for many diseases.[5] Whether oxidative stress itself is a cause or a consequence of disease is a well-researched question with few clear answers to date.[5] One strategy for answering this question is to identify biochemical changes that result from oxidative stress and differentiate between those that truly reflect tissue injury and those that are adaptations invoked to boost cellular defense systems.

We have previously shown that a candidate protein with the potential to contribute to the cellular adaptation to oxidative stress is phospholipase A_2 (PLA_2, EC 3.1.1.4). The PLA_2 constitute a family of enzymes—distinguished by size, cellular location, calcium dependence, and substrate preferences—that play pivotal roles in cellular metabolism.[6,7] The PLA_2 catalytic activity is enhanced in the presence of ROS by mechanisms that involve catalytic preference for oxidized substrate and/or activation in response to phosphorylation.[8–10] Using a model of dietary deficiency that leads to chronic oxidative stress, we have previously shown that the calcium-independent (Ca^{2+}-independent) PLA_2 activity in the lung and liver was 2 to 6 fold higher in rats maintained for 6 weeks from weaning on torula yeast-based diets deficient in vitamin E and selenium compared with the tissue of control rats maintained on diets adequate in these two nutrients.[11] These experiments further characterize the effect of vitamin E and Se deficiency on Ca^{2+}-independent PLA_2 activity in an additional rat tissue (the spleen), and compare the time course of the enhanced Ca^{2+}-independent PLA_2 activity in lung, liver, and spleen. These three tissues were chosen because all exhibit dramatic increases in Ca^{2+}-independent PLA_2 activity in response to deficiency of vitamin E and Se. The results help to further define the utility of the vitamin E and Se dietary deficiency model for studying cellular adaptations to oxidative stress.

Methods and materials

Experimental design

In experiment protocol A, weaning (40–50g) Long-Evans hooded rats (Harlan, Indianapolis, IN, USA) (n = 32) were randomly assigned to torula yeast-based diets adequate in vitamin E (100 mg α-tocopherol equivalents/kg diet) and selenium (0.228 mg Se/kg diet), deficient in vitamin E (<0.1 mg/kg diet), deficient in selenium (<0.025 mg Se/kg diet), or deficient in both nutrients, yielding four dietary treatment groups (+E, +Se; −E, +Se; +E, −Se; −E, −Se). The diet composition was previously described.[11] The rats were fed the treatment diets for 6 weeks, at which time they were anesthetized with ketamine (90 mg/kg): xylazine (10mg/kg), killed by exsanguination, and blood and tissues taken for analysis as previously described.[11]

To determine the time frame of enhanced Ca^{2+}-independent PLA_2 activity, in experiment protocol B, weanling Long Evans hooded rats were randomly assigned to either diets sufficient in both vitamin E and Se (+E, +Se) or deficient in these two nutrients (−E, −Se). The rats consumed these two diets for up to 7 weeks. After 6 weeks four animals that were consuming the −E, −Se diet were switched to the +E, +Se diet and maintained for an additional week (replete +E, +Se). Blood samples were taken at 1 and 2 weeks. Beginning at 3 weeks and continuing at weekly intervals throughout the study, rats were killed (4 to 8 per week), and the lungs were perfused with physiological saline until cleared of blood. The tissues were removed, frozen in liquid N_2, and stored at −80°C until used. The time course experiments were conducted on two different occasions with 34 rats in each study.

Tissue preparation

Tissue samples were thawed on ice, weighed, and homogenized (10%, w/v) in 25 mmol/L tris-HCl pH 7.4 containing 250 mmol/L sucrose and 1.0 mmol/L EDTA. Homogenates were centrifuged to obtain cytosol and 100,000 × g pellet. The microsomal pellet was resuspended in 25 mmol/L tris-HCl pH 7.4 containing glycerin (100 g/L) and 1 mmol/L EDTA.

Vitamin E and selenium analysis

Vitamin E as α-tocopherol was determined in plasma, lung and liver 100,000 × g pellet, and spleen homogenates. Sample extraction was carried out as described by MacCrehan,[12] and tocopherols were separated by HPLC and quantified by electrochemical detection by the procedure of Pascoe et al.[13] Selenium status was assessed by measuring Se-dependent glutathione peroxidase (GSH-Px) activity spectrophotometrically at 340nm.[11] The assay mixture contained 100 mm/L potassium phosphate buffer pH 7.0, 2 mmol/L EDTA, 1 mmol/L sodium azide, 1 mmol/L GSH, 0.2 mmol/L NADPH, 3 units GSH-reductase, and sample (10–100 μg protein). The reaction was initiated by the addition of the hydrogen peroxide (0.25 mmol/L final concentration) and the oxidation of NADPH ($\epsilon = 6.2 \times 10^3$ $Lmol^{-1}$ cm^{-1}) monitored at room temperature for 2 minutes.

Determination of PLA_2 activity

Phospholipase A_2 activity was determined in lung, spleen, and liver cytosolic samples using L-α-dipalmitoyl[2-palmitoyl-9,10-^{3}H] phosphatidylcholine (DPPC) (specific activity 1850 GBq/mmol, New England Nuclear, Boston MA) as substrate. Unlabeled substrate (0.8 mmol/L) was dispersed by sonication in 100 mmol/L 2-[N-morpholino]ethanesulfonic acid (MES) buffer pH 6.5 containing 5 mmol/L $CaCl_2$ and 0.05% Triton X-100. The cold substrate solution was added to the radiolabeled phosphatidylcholine to give a final specific activity of 25.9 MBq/mmole and resonicated until the solution was clear. Aliquots of sample (50 μL) were equilibrated at 37°C for 2 minutes before the addition of substrate solution (50 μl, final concentration 0.4 mmol/L).

Incubations were conducted up to 60 minutes, and the reaction was terminated by the addition of 1 mL of extraction solvent (2-propanol:heptane:0.5 M sulfuric acid, 4:1:0.1) and vortexed. The phases were separated and clarified by the successive addition of 1.4 mL of heptane and 0.4 mL of distilled H_2O followed by centrifugation at 600 xg for 5 min. The organic layer was removed into 1 mL of heptane containing 150 mg silica gel 60 (Baxter Scientific Products, Mcgaw Park, IL, USA), vortexed, and centrifuged. An aliquot of the supernatant was removed and radioactivity determined by liquid scintillation counting. Recovery was determined using [^{14}C]-palmitic acid (ICN Biomedicals, Inc., Irvine, CA, USA). Non-enzymatic hydrolysis was determined and subtracted from the enzymatic rate. Activity is expressed as nmoles fatty acid released $min^{-1} \cdot L^{-1}$, and specific activity determined by dividing the activity for each sample by the protein concentration. Calcium-independent activity was determined as described in buffer containing 5 mmol/L EGTA instead of $CaCl_2$. Calcium-dependent activ-

ity was calculated by subtracting the calcium-independent activity from the total activity for each sample.

To determine whether phospholipase C activity was contributing to fatty acid release in the PLA_2 assay, a time course experiment was conducted using pooled lung cytosol (200 μg) obtained from rats consuming the +E, +Se and –E, –Se diets for 7 weeks. These two samples were incubated with the substrate and the amounts of palmitic acid and diacylglycerol formed in the reaction mixtures was determined during a 60-min period. The incubations were conducted as described, except that aliquots (100 μL) were extracted at timed intervals using 1.9 mL of chloroform:methanol and 0.4 mL of physiological saline containing 0.01 N HCl. The organic layer was removed, dried under N_2, and spotted onto a TLC plate (LK5D silica gel, Whatman, Chilton, NJ, USA). The plate was developed in petroleum ether:diethyl ether:acetic acid (70:30:1). Palmitic acid (Rf = 0.60) separated from diacylglycerol (Rf = 0.40) in this system. The bands corresponding to each lipid product and the PC substrate (origin) were scraped and radioactivity determined by liquid scintillation counting.

Malondialdehyde measurement

Total malondialdehyde in rat lung and spleen homogenates was measured by the HPLC procedure previously described.[11]

Protein concentration

Protein was determined by the bicinchoninic acid method[14] using reagents supplied by Pierce (Rockford, IL, USA).

Statistics

Data from experiment protocol A were analyzed by two-way analysis of variance (ANOVA) with the SigmaStat software package (Jandel Scientific, San Rafael, CA, USA) using the main effects of Se and vitamin E status. When a significant F was obtained, differences between group means were assessed by Student-Newman-Keuls.[15] Data from experiment protocol B were analyzed by multiple linear regression with the independent variables diet, time, and study (representing the two different occasions that the diet study was conducted). Finding no significant effect of experiment, the data were pooled and tested for time effects by one-way ANOVA, and for dietary treatment effects at each time point by either Student's *t* test (weeks 3 through 6) or one-way ANOVA with Student-Newman-Keuls as the post hoc test (week 7). A $P < 0.05$ was considered to be statistically significant.

Results

Protocol A. Spleen phospholipase A_2 activity.

We have previously shown that rats maintained on vit E and selenium deficient diets for 6 weeks from weaning have 2 to 10 fold higher Ca^{2+}-independent PLA_2 activity in lung and liver compared with animals that consume comparable diets sufficient in these nutrients (control) or deficient in either nutrient alone.[11] This response to the deficiency of both vitamin E and selenium was not observed with heart or kidney tissue. Also, no effect of dietary treatment was observed with rat serum (data not shown). The results of analysis of spleen cytosol are presented in *Figure 1*. Spleen cytosol from rats consuming the experimental diets for 6 weeks had approximately 5 fold higher Ca^{2+}-independent PLA_2 activity in –E, –Se samples compared to control and single nutrient deficient samples ($P < 0.05$ for Se, vitamin E, and interaction). In addition, spleen cytosol was found to exhibit about 6 fold greater calcium-dependent PLA_2 activity in –E, –Se samples compared with the other three groups ($P < 0.05$ for Se, vitamin E, and interaction).

Protocol B. Time course of changes in calcium-independent phospholipase A_2 activity with vitamin E and Se deficiency.

The effect of the duration of consumption of the vitamin E and selenium deficient diet on rat lung, spleen, and liver cytosolic Ca^{2+}-independent PLA_2 activity is presented in *Figure 2*. These 3 tissues were chosen because each exhibited significantly higher Ca^{2+}-independent PLA_2 activity in rats consuming the double deficient diet compared with rats consuming the control diet or diets deficient in either vitamin E or Se. In lungs both diet ($P = 0.0018$) and time effects ($P < 0.001$) were observed. Pairwise comparisons revealed that Ca^{2+}-independent PLA_2 activity was not different at 3 weeks, but was 3 fold higher in –E, –Se lung cytosols at 4 through 7 weeks of consuming the diet compared with the +E, +Se lung cytosols ($P < 0.05$). Rats partially replete with the +E, +Se diet for 1 week (replete +E, +Se) had Ca^{2+}-independent PLA_2 activity that was not different from animals maintained on the control diet for 7 weeks. Animals consuming the +E, +Se diet had comparable Ca^{2+}-independent PLA_2 activity during weeks 3 through 6, and at 7 weeks the activity was not significantly

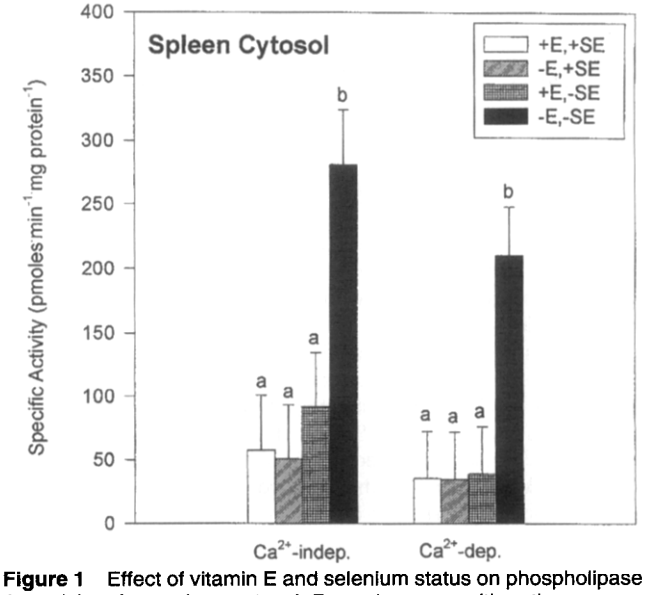

Figure 1 Effect of vitamin E and selenium status on phospholipase A_2 activity of rat spleen cytosol. Bar values are arithmetic means ± SD, $n = 6–8$. Bars within a grouping not sharing a letter are significantly different ($P < 0.05$).

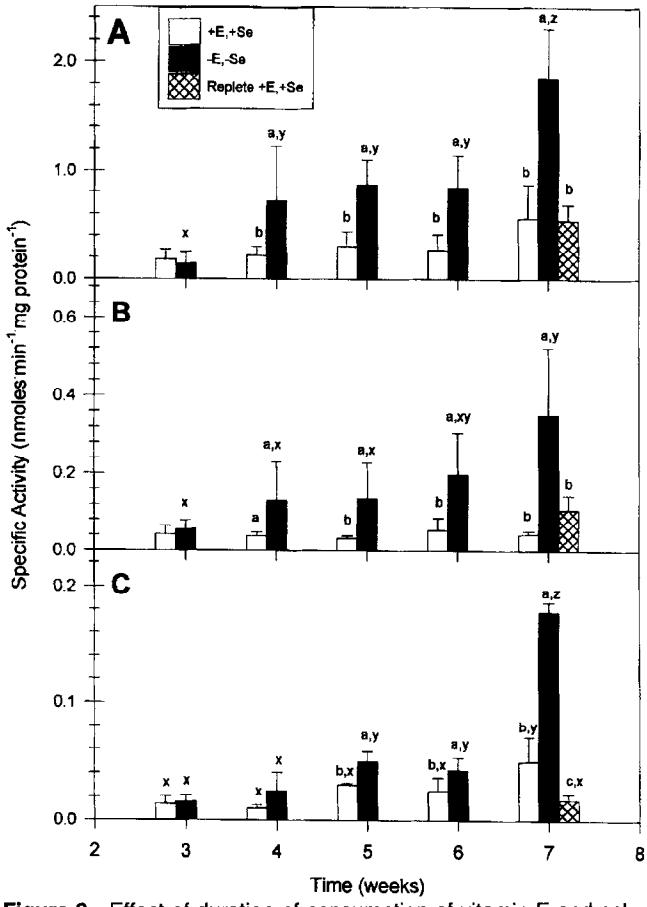

Figure 2 Effect of duration of consumption of vitamin E and selenium deficient diet on calcium-independent phospholipase A_2 activity of rat lung (A), spleen (B) and liver (C) cytosol. Bar values are arithmetic means ± SD, n = 4–8. ^{a,b}Indicates a significant difference between means with respect to diet ($P < 0.05$). ^{x,y,z}Indicates a significant difference between means with respect to time ($P < 0.05$).

higher ($P = 0.15$) than that measured for the previous weeks. Rats consuming the –E, –Se diet had 3 fold higher Ca^{2+}-independent PLA_2 activity at weeks 4, 5, and 6 than at week 3 ($P < 0.05$) and sixfold higher activity at week 7. Thus, there appears to be a bi-phasic response in Ca^{2+}-independent PLA_2 activity to the deficiency of vitamin E and Se with the first phase occurring during weeks 4 to 6 and the second occurring at the seventh week.

Results similar to the lung cytosols were obtained with both spleen and liver cytosols (*Figure 2 B & C*). In these tissues both diet ($P < 0.01$) and time effects ($P = 0.05$) were observed. The graphs of the results of the three tissues are almost identical. However, several differences were noted. In spleen, although mean values were higher in the –E, –Se group compared with the +E, +Se at earlier time points, no statistically significant differences were observed until week 6. In liver, statistically higher Ca^{2+}-independent PLA_2 activity was observed starting at 5 weeks of consuming the –E, –Se diet.

The results of the effect of duration of consumption of the experimental diets differed slightly among the three tissues. For rats consuming the +E, +Se diet, no increase in Ca^{2+}-independent PLA_2 activity was observed at 7 weeks in lung and spleen, whereas a significant increase was observed in liver. For the spleen of rats consuming the –E, –Se diet, one-way ANOVA analysis did not reveal a difference among the means at weeks 3 through 6. However, at 7 weeks the mean Ca^{2+}-independent PLA_2 activity was higher than the 3, 4, and 5 week time points ($P < 0.05$). For the liver of rats consuming the –E, –Se diet, one-way ANOVA analysis did not reveal a difference between the mean Ca^{2+}-independent PLA_2 activities at week 4 in comparison with week 3, but activity was 2 fold higher at weeks 5 and 6, and over 5 fold greater at week 7 ($P < 0.05$).

To verify that the assay procedure used in these experiments was measuring Ca^{2+}-independent PLA_2 activity and not phospholipase C activity, a timed incubation experiment was conducted using pooled rat lung cytosol as a source of enzyme (*Figure 3*). The potential products of the reaction, free palmitic acid, and diacylglycerol, were separated and quantified as described under Methods and materials. For incubations containing either +E, +Se or –E, –Se samples the amount of palmitic acid in the reaction mixture increased in a linear manner from initiation through 10 min. Thereafter the amount of free fatty acid continued to increase up to 60 min. Conversely, no appreciable increase in diacylglycerol was observed in either incubation until after 10 min after the initiation of the reaction. The results of this experiment verify that the assay procedure reported here quantified Ca^{2+}-independent PLA_2 activity in rat tissue cytosol and not phospholipase C activity.

Glutathione peroxidase activity of blood, lung, and spleen

The results of the time course studies on glutathione peroxidase activity of lung, spleen, and liver cytosols are presented in *Figure 4 A, B, & C*. Whole blood glutathione

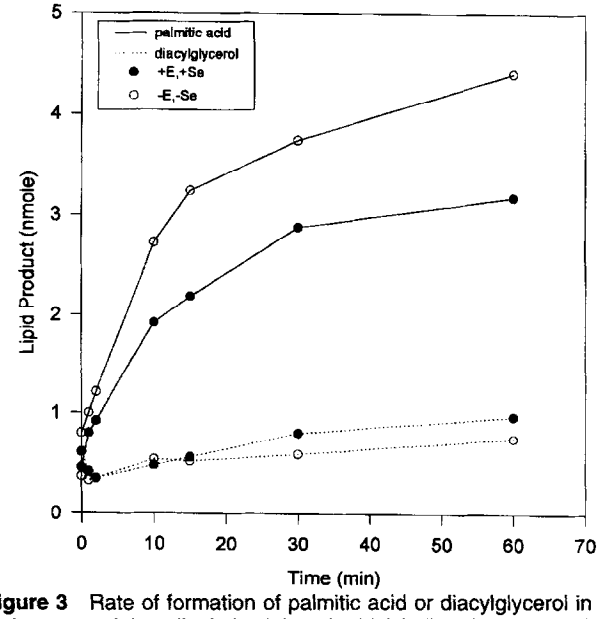

Figure 3 Rate of formation of palmitic acid or diacylglycerol in reactions containing dipalmitoylphosphatidylcholine (0.4 mmol/L) as substrate and pooled lung cytosol (200 µg) from either +E, +Se or –E, –Se rats.

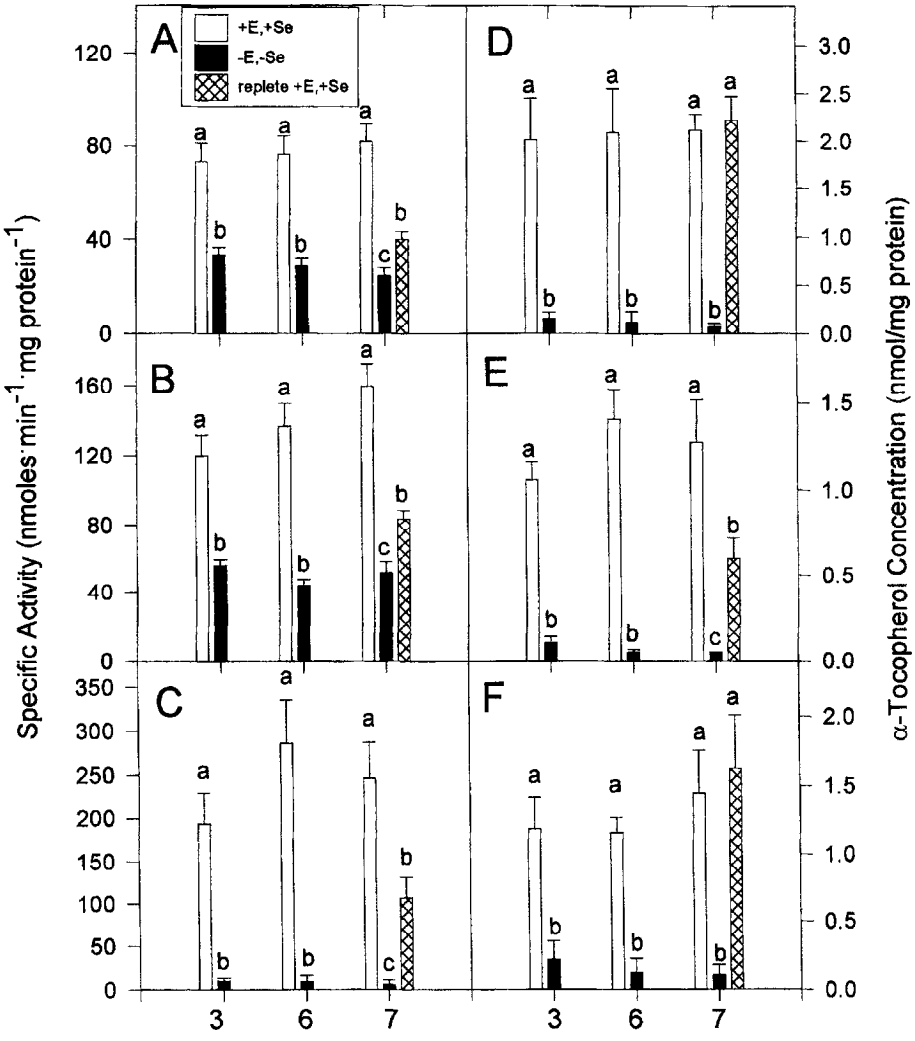

Figure 4 Effect of duration of consumption of vitamin E and selenium deficient diet on the Se-dependent glutathione peroxidase activity of rat lung (A), spleen (B), and liver (C) cytosol, as well as α-tocopherol concentration of rat lung (D) and liver (F) 100,000 × g pellet and spleen homogenate (E). Bar values are arithmetic means ± SD, $n = 4$–8. ^{a,b,c}Indicates a significant difference between means with respect to diet ($P < 0.05$).

peroxidase activity was not different between the two groups after 1 week of consuming the diet, but began to diverge at 2 weeks and appeared to reach a minimum in the −E, −Se group by about 3 weeks of consuming the diet—averaging between 25 to 30% of the mean for the +E, +Se activity for the duration of the experimental treatment (data not shown). Similar results were obtained for the lung and spleen cytosol at 3 through 7 weeks with diet being the only significant predictor of activity ($P < 0.01$). In liver, glutathione peroxidase activity of rats consuming the −E, −Se diets reached a minimum at 3 weeks and averaged 5% or less of the activity of the +E, +Se rats throughout the rest of the experimental time period ($P < 0.01$). Consumption of the control diet by deficient rats (replete +E, +Se) for 1 week raised glutathione peroxidase activity in all three tissues to 50% of the activity measured for rats consuming the +E, +Se diet for 7 weeks ($P < 0.05$).

Vitamin E concentration of plasma, lung, and spleen

The results of the time course studies on α-tocopherol concentration of lung and liver 100,000 × g pellet and spleen homogenates are presented in *Figure 4 D, F & E*. The time course of vitamin E depletion in these rats exhibited a similar trend with respect to duration of consumption of the deficient diet as that observed for glutathione peroxidase activity. Plasma α-tocopherol content reached a minimum in the −E, −Se group by 3 weeks of consuming the diet and averaged 3 to 5% of the values for the +E, +Se group thereafter (data not shown). This was also the trend observed for the lung and liver 100,000 × g pellet, and the spleen homogenate, again with diet being the only significant predictor of α-tocopherol concentration ($P < 0.01$). Consumption of the control diet by deficient rats (replete +E, +Se) for 1 week raised the vitamin E content of all three

tissues. In lung and liver 100,000 × g pellet the α-tocopherol concentration was the same in the replete +E, +Se as rats consuming the control diet for 7 weeks. In spleen homogenates the trend was the same as that observed with glutathione peroxidase activity—1 week repletion with vitamin E raised the α-tocopherol concentration to 50% of that of the rats consuming the +E, +Se diet ($P < 0.05$).

Malondialdehyde concentration

The amount of malondialdehyde (MDA) was analyzed, as a qualitative marker of lipid peroxidation, in the lung, spleen and liver tissue obtained from rats that had consumed the experimental diets for 6 and 7 weeks (*Figure 5*). Precautions were taken in analyzing the tissues to assure that the results obtained would reflect MDA formation in vivo and not an artifact of sample processing. The freshly extracted tissues were frozen in liquid nitrogen, stored at −80°C until the day of analysis, and then homogenized and analyzed immediately. In lung, although a trend towards higher MDA in the −E, −Se homogenates was observed at the two time points no statistically significant differences were obtained ($F = 1.83$). In spleen, no differences were observed at the 6-week time point, but at 7 weeks the MDA concentration in the −E, −Se samples was approximately 60% higher than the +E, +Se samples ($P < 0.05$), but not different than the replete +E, +Se. In liver, no statistically significant differences were observed.

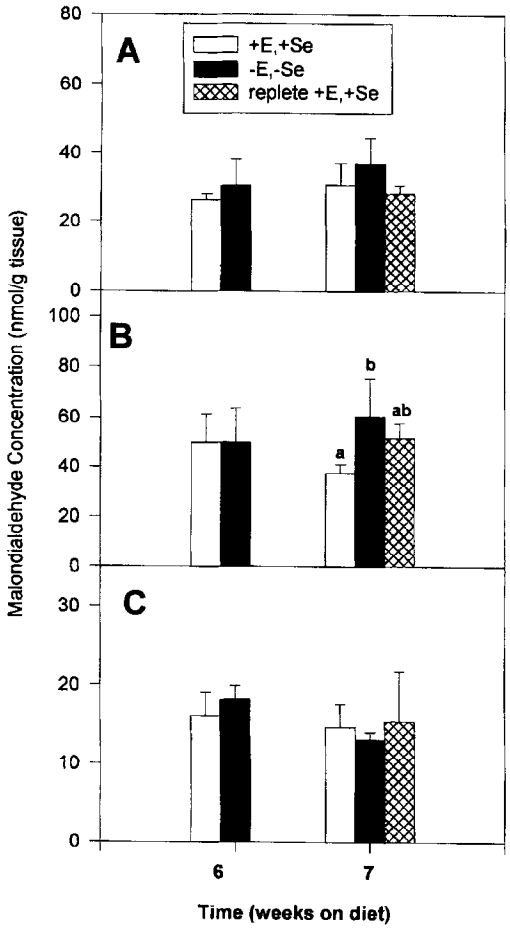

Figure 5 Effect of vitamin E and selenium deficiency and repletion on the malondialdehyde concentration of rat lung (A), spleen (B), and liver (C) tissue of rats consuming the experimental diets for 6 and 7 weeks. Bar values are arithmetic means ± SD, $n = 4–8$. [a,b]Indicates a significant difference between means with respect to diet ($P < 0.05$).

Pearson correlations

Correlations between individual sample results for the measured parameters are presented in *Table 1*. The number of samples differs for each analysis based on the frequency of use of samples from the same animal. As expected, negative correlations were observed between PLA_2 activity (lung and spleen) and glutathione peroxidase activity, and PLA_2 activity and vitamin E concentrations (lung, spleen, and liver). The correlation coefficients ranged from −0.395 to −0.544. Positive correlations were observed between PLA_2 activity results for all three tissues, and between PLA_2 activity and MDA concentrations. For the latter analysis correlations were significant ($P < 0.05$) for lung and spleen, but not liver. These results support an association between enhanced Ca^{2+}-independent PLA_2 activity and increased lipid peroxidation in rat lung and spleen.

Discussion

The purpose of these studies was to define the time frame of the response of the Ca^{2+}-independent PLA_2 to dietary deficiency of vitamin E and Se as a means to determine whether this deficiency model might provide insight into biochemical adaptations to oxidative stress. Additionally, this work is a further characterization of the tissue response of PLA_2 to dietary deficiency of vitamin E and Se.

When weanling rats are maintained on diets deficient in both vitamin E and selenium, dietary necrotic liver degeneration develops,[16] and can lead to death in a short time.[17] Survival is usually 4 to 5 weeks when the consumed diets are low in methionine. However, rats consuming deficient diets containing supplemental methionine, as is the case with the diets used in these experiments, survive for a longer period, and can show both hepatic and pulmonary necrosis as well as hematuria at the time of death.[18] Survival time can also vary considerably based on the weight of the animals at the time of initiation of the study. For instance, Chow[19] reported on rats weighing an average of 125 grams

Table 1 Pearson product correlations between calcium-independent phospholipase A_2 (PLA_2) activity and glutathione peroxidase activity (GPx), α-tocopherol concentration (Vit E), and malondialdehyde (MDA) concentration of rat lung, spleen and liver

	Tissue Measurement			
	GPx Activity	Vit E	MDA	Lung PLA_2
Lung PLA_2	-0.525^c	-0.395^a	0.545^a	—
	$n = 44$	$n = 29$	$n = 15$	
Spleen PLA_2	-0.400^a	-0.544^a	0.633^b	0.702^c
	$n = 41$	$n = 19$	$n = 17$	$n = 47$
Liver PLA_2	NS	-0.472^b	NS	0.835^c
		$n = 28$		$n = 42$

[abc]Statistical significance ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$), NS - not significant.

that consumed deficient diets for 105 days with no mortality. Awad et al.[20] recently reported on weanling rats that were maintained on deficient diets with no mortality until after 12 weeks. In the experiments reported here we see no mortality up to 5 weeks of consuming the –E, –Se diet, a low mortality rate during the sixth week (5.0%, 2 out of 40), and this increases during the seventh week (10%, 1 out of 10). The diets used in these experiments are similar in composition to those reported previously,[20] except the current diets were higher in fat content: 16% (10% corn oil and 6% lard) versus 6.7% (all corn oil). Thus, it would appear that polyunsaturated fat composition of the diet also affects survival outcome of rats maintained on vit E and Se deficient diets, and this is likely to also affect the magnitude and time of onset of biochemical changes that result from deficiency of these nutrients.

Characterizing the time frame of biochemical response to vitamin E and Se deficiency is a useful endeavor to help differentiate from changes that are adaptive and protective from those that result from tissue injury. Previous work has documented changes in several key cellular enzymes that play important roles in metabolism and detoxification in response to deficiency of vitamin E and/or selenium in the rat. Selenium deficiency has been shown to elevate the activity of the glutathione S-transferases (GST),[21,22] prostaglandin dehydrogenase,[22] and enzymes involved in xenobiotic metabolism.[23] Selenium deficiency leads to a 2 fold increase in protein and message for rat liver GST isozymes with non-selenium-dependent glutathione peroxidase activity.[24] Selenium deficiency has also been shown to lead to elevation of plasma glutathione concentrations.[25] The time frame for enhancement in plasma GSH levels with Se deficiency in the previous report[25] appears similar to the time frame reported here for enhanced Ca^{2+}-independent PLA_2 activity in lung and spleen with combined vitamin E and Se deficiency.

Previous work has documented the effects of combined vitamin E and selenium deficiency on markers of tissue injury and impaired cellular function. In both chickens and rats deficiency of both nutrients is associated with impaired immune function.[26,27] Evidence of liver necrosis,[28] impaired kidney function,[29] muscle breakdown,[19] and lung injury[30] have also been documented. Biochemical markers have been used to characterize these changes including: elevated plasma lactate dehydrogenase and glutamate-oxaloacetate transaminase for liver necrosis,[31] increased clusterin expression for kidney injury,[29] and increased plasma pyruvate kinase activity reflecting myodegeneration.[19] Other changes that have been documented previously include elevation of markers of lipid peroxidation such as ethane exhalation,[32] liver MDA levels,[19] and F_2-isoprostane concentrations in a variety of tissues.[20] In these studies we measured the level of MDA in lung, spleen, and liver obtained from the rats consuming diet for 6 and 7 weeks. Unlike our previous report[11] we did not find statistically significant differences in the lung at either time point, but did find a higher level in the spleen of –E, –Se animals at seven weeks compared to the +E, +Se. Chow reported higher MDA equivalents in the liver of rats receiving the –E, –Se diet for 12.3 weeks compared to rats receiving diets adequate in both nutrients.[19] But, recently Diplock et al.[33] reported undetectable levels in liver microsomes from rats consuming vitamin E and Se deficient diets for 4 weeks. Both differences in the amount of time animals consume the diets and a lack of specificity of MDA as a marker of lipid peroxidation[34] are plausible explanations for the discrepancy in the results of the different studies. Although we did find significant correlations between Ca^{2+}-independent PLA_2 activity and MDA concentrations in lung and spleen, other markers of lipid peroxidation might provide better indicators of the relationship between enhanced PLA_2 activity and the degree of oxidative stress. Recent evidence has suggested that another factor to consider in choosing a marker of lipid peroxidation is its rate of metabolism in vivo. Thus, F_2-isoprostanes may be a better marker of lipid peroxidation than MDA because of greater stability,[35] and the effect of dietary deficiency on metabolizing enzymes.[36]

The biochemical changes associated with the development of vitamin E and selenium deficiency in rats may provide a good model for studying the progression of changes that occur as ROS accumulate in cells—from early events that may involve adaptations, to defense against oxidative stress, to the injury that results from accumulating tissue damage. The PLA_2 response in this system may be especially useful to study the mediators of this progression. Enhanced PLA_2 activity may either be protective—leading to increased metabolism of hydroperoxy fatty acids,[37] or deleterious—further propagating the lipid peroxidation chain reaction.[38] Borowitz and Montgomery[39] found that activation of PLA_2 by treatment of rat liver microsomes with mellitin before initiation of lipid peroxidation led to decreased levels of thiobarbituric acid reactive substances (TBARS) and conjugated dienes, suggesting a protective role for PLA_2. Activation of PLA_2 after stimulation of lipid peroxidation showed no effect. Conversely, inhibition of PLA_2 during lipid peroxidation decreased the level of TBARS but not conjugated dienes, which suggests that an active PLA_2 further propagates lipid peroxidation and is deleterious to the cell. In models of acute oxidative stress that reflect disease conditions PLA_2 activation has been found to be associated with tissue injury. Al-Mehdi and coworkers[40] found that inhibition of PLA_2 decreased the amount of TBARS generated during ischemia-reperfusion of the whole rat lung, as well as during t-butyl hydroperoxide-induced lipid peroxidation of lung microsomes. Hazen and coworkers[41] have reported that the 10 fold increase in Ca^{2+}-independent PLA_2 activity that occurs during experimental myocardial ischemia is responsible for the accelerated phospholipid breakdown that is observed in that system. Thus, enhancement in PLA_2 activity may have different cellular consequences depending on the degree of oxidative stress, the timing of the activation event, and the form of the enzyme that is activated.

The forms of Ca^{2+}-independent PLA_2 that are responding to vitamin E and Se deficiency in this model are not yet clear. Three Ca^{2+}-independent PLA_2 have been characterized to date. The first are plasmalogen-specific PLA_2s, which include a 40 kDa cytosolic form that has been isolated and characterized from canine and human heart,[42,43] and a membrane-associated form that is activated 10 fold during experimental myocardial ischemia.[41] A second Ca^{2+}-

independent PLA_2 is a form of the enzyme that has been purified recently from lung cytosol and is reported to be associated with the lysosomal fraction.[44] This form of PLA_2 is active at low pH (4.0), and is implicated in surfactant metabolism.[45] A third distinct form of Ca^{2+}-independent PLA_2 has been purified from the macrophage-like cell line $P388D_1$[46] and shows a lack of specificity for acyl or alkyl-ether containing phospholipids. The native form of this enzyme is thought to exist as a large oligomeric complex. Further work is underway to determine whether any of these forms of Ca^{2+}-independent PLA_2 are enhanced by vitamin E and Se deficiency.

In summary, combined vitamin E and Se deficiency in rats leads to enhanced Ca^{2+}-independent PLA_2 activity in lung, spleen and liver cytosol. The time frame of this increase in activity was found to be bi-phasic with the initial increase occurring between 4 and 6 weeks of consumption of the deficient diet, followed by a further increase at 7 weeks. The initial enhancement could be reversed by partial amelioration of the deficient state. The malondialdehyde concentration of lung and spleen measured at 6 and 7 weeks correlated positively with the Ca^{2+}-independent PLA_2 activity. Further work is underway to characterize the forms of Ca^{2+}-independent PLA_2 that are enhanced by the deficiency of vitamin E and Se in rats.

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