Stabilization of Hepatic Lysosomes of Rats by Vitamin E and Selenium in vivo as Indicated by Thermal Labilization of Isolated Lysosomes

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ABSTRACT An in vivo system was used to study the effects of vitamin E, selenium (in the form of sodium selenite) and steroidal antiinflammatory drugs on hepatic, lysosomal membranes. After administering these compounds for 3 days to rats, hepatic lysosomes were isolated and subjected to thermally induced labilization by incubating lysosomes in 0.25 M sucrose at 45° for 90 minutes. Release of two enzymes, acid phosphatase and β-glucuronidase, was used as an index of labilization. Vitamin E stabilized lysosomes in dosages of 0.100 to 3.9 units/kilogram and produced maximal stabilization at a daily dosage of 0.100 unit/kilogram. Selenium was effective in stabilizing lysosomes at a dose level of 2.0 µg/kg, only. Low dosages of selenium (0.5 µg/kg) potentiated vitamin E (0.039 unit/kg), but higher dosage (50 µg/kg) antagonized vitamin E (3.4 units/kg). Results suggest that the interaction of vitamin E and selenium in attenuating animal diseases caused by deficiencies of vitamin E or selenium may be due to their mutual potentiation in stabilizing lysosomal membranes, provided that they are administered in the same ratio and at the low levels found herein to cause such potentiation. J. Nutr. 102: 1413–1420, 1972.

INDEXING KEY WORDS vitamin E • selenium • liver lysosomes

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1 A preliminary report of results with vitamin E was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Palo Alto, California, August, 1970, in a panel discussion on lysosomes and in a formal presentation.

2 Taken in part from a dissertation to be presented in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

The mode of interaction of selenium and vitamin E in preventing experimentally induced diseases in animals is unknown, but the activities of both substances seem to be closely associated (1–6). Thus, rats fed a necrogenic diet develop hepatonecrotic lesions characterized by progressive degeneration of hepatocellular plasma membranes (1); dietary supplements of vitamin E or selenium prevent such necrosis (1) suggesting that either agent stabilizes the hepatocellular plasma membrane. Vitamin E also stabilizes erythrocyte and leucocyte membranes during storage of human blood, thereby preserving the normal morphology of erythrocytes and attenuating the decrease in the number of leucocytes (7). Degenerative changes in Duchenne’s muscular dystrophy (8), vitamin E or selenium deficiencies (9–12) and white muscle disease (9–12) can be correlated to some degree with the release of lysosomal enzymes. In the former disease, enzymes are apparently released from cells of cardiac and skeletal muscles, liver cells, and erythrocytes (8), suggesting a generalized lability of the membranes of all of these types of cells. Thus, a general, non-specific, membrane-stabilizing effect of selenium and/or vitamin E could explain their prophylactic and curative (12, 13) effects, especially if one considers that a stabilizing effect may be applicable to the membranes of endothelial cells of the microvasculature, lysosomal membranes, mitochondrial membranes, and cellular membranes of the liver, muscle, pancreas (6) and other tissue cells.

The purpose of the present investigation was to determine whether selenium and/or vitamin E have a protective effect in vivo...
on lysosomal membranes. This was estimated quantitatively by administering these substances to rats for 3 days, isolating their hepatic lysosomes and subjecting the latter to thermal labilization (14, 15).

MATERIALS AND METHODS

Enrichment of lysosomes. Drugs or vehicle were administered to normal rats of the Sprague-Dawley strain (male, 175 to 250 g, 43 to 57 days old) intraperitoneally for 3 days and the animals were killed on the morning of day 4. Animals were fed a commercial stock diet\(^3\) (65 IU vitamin E/kg) ad libitum. Experiments with vitamin E were carried out over a period of 2 years. Results with the two higher dose levels were repeated two or three times for confirmation of results both in this and another laboratory.\(^4\) Results with control animals were obtained repeatedly over the 2-year period using four to eight animals per experiment. Thus animals were not fed a single supply of stock diet.

The enrichment of lysosomes and all other procedures were similar to those previously described (14, 15). Briefly, portions of livers from two animals (\(N = 1\)) were pooled, minced and immediately placed in ice-cold (\(2^\circ\)) 0.25 M sucrose. Thus, for \(N = 4\) in table 1, eight drug-treated rats would have been used. A 10% homogenate was prepared by grinding briefly 3 g of liver in a mortar containing ice-cold 0.25 M sucrose followed by further homogenization in a motor-driven Potter-type tissue homogenizer (Teflon pestle) using three to four up and down strokes. The volume of the resulting homogenate was adjusted to 30 ml, divided into two equal portions in two graduated cylinders and each aliquot was centrifuged (1000 \(X\) g, \(4^\circ\), 15 minutes) to remove unbroken cells and nuclear debris. The supernates from the two centrifuge tubes were recovered and centrifuged (25,000 \(X\) g, \(4^\circ\), 15 minutes) to obtain the lysosomal-rich pellets. This preparation thus contained mitochondria. The pellet in one centrifuge tube was resuspended in ice-cold 0.25 M sucrose (15 ml) and incubated at 22\(^\circ\) for 30 minutes to obtain maximal hypotonically induced release of lysosomal enzymes. The pH of the resulting suspension was 6.8 due to endogenous buffer systems. Detergents such as Triton X-100 were not employed to disrupt lysosomes because in preliminary experiments it could be demonstrated that lysosomal membranes obtained after centrifugation (25,000 \(X\) g, \(4^\circ\), 15 minutes) of hypotonically disrupted lysosomes are disintegrated by such agents, thereby releasing membrane-bound enzymes. After incubation, each tube was centrifuged (25,000 \(X\) g, \(4^\circ\), 15 minutes) and the supernates were assayed for released enzymes. Released enzyme activity was calculated by dividing the amount of enzyme activity released thermally by the amount released in distilled water, times 100. Percentage enzyme release of drug-treated animals was compared to that of vehicle-treated animals and percentage inhibition of release due to drug effects was calculated therefrom. Total enzyme activity released hypotonically was not decreased by treatment with any of the drugs reported herein. Selenium was administered in the form of sodium selenite.

Enzyme assays. Acid phosphatase (AP, EC 3.1.3.2) and \(\beta\)-glucuronidase (BG, EC 3.2.1.31) activities were determined using modifications of methods previously described (14, 15). Briefly, the reaction mixture for AP contained 0.5 ml of 0.09 M citrate buffer solution, pH 4.8, 0.2 ml \(p\)-nitrophenyl phosphate (10 mg/ml), 0.3 ml distilled water and 0.05 ml of lysosomal enzyme solution. Incubation was carried out at 37\(^\circ\) for 30 minutes and was terminated by the addition of 5.0 ml of 0.1 N NaOH. The absorbance (OD) of the resulting solution was measured at 420 m\(\mu\) using a colorimeter.\(^5\) The reaction mixture for BG contained 0.5 ml of 0.075 M phosphate buffer, pH 4.5, 0.5 ml of 0.0015 M phenolphthalein glucuronic acid, 0.2 ml distilled water, and 0.15 ml of enzyme solution. After 30 minutes' incubation at 37\(^\circ\), reaction was terminated by addition of 5.0 ml

\(^3\) Content of vitamin E in stock diet (Purina Laboratory Chow) supplied by manufacturer. Manufacturer neither analyzes for selenium nor knows its content in stock diet.

\(^4\) Unpublished data, Lyle Branch, University of Mississippi.

\(^5\) Bausch and Lomb Spectronic 20 Colorimeter.
HEPATIC LYSOSOMES, VITAMIN E AND SELENIUM 1415

TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Daily dose (mg/kg ip)</th>
<th>N</th>
<th>AP (%)</th>
<th>BG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E</td>
<td>0.039</td>
<td>7</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>3</td>
<td>53</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>0.390</td>
<td>7</td>
<td>49</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>3.900 *</td>
<td>10</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>Se</td>
<td>0.0005</td>
<td>4</td>
<td>5</td>
<td>+10</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>4</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>0.008 *</td>
<td>4</td>
<td>19</td>
<td>+14</td>
</tr>
<tr>
<td>Vitamin E + Se</td>
<td>0.039 + 0.0005</td>
<td>4</td>
<td>30</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>0.039 + 0.002</td>
<td>4</td>
<td>21</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>0.340 + 0.005</td>
<td>3</td>
<td>43</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>3.400 + 0.050</td>
<td>3</td>
<td>+63</td>
<td>+59</td>
</tr>
<tr>
<td></td>
<td>34.000 + 0.500</td>
<td>3</td>
<td>+16</td>
<td>+22</td>
</tr>
</tbody>
</table>

1 Units per kilogram vitamin E as d-a-tocopheryl acetate; one unit vitamin E equals 0.67 mg d-a-tocopherol.

2 AP = acid phosphatase; BG = β-glucuronidase.

3 P < 0.05 compared to Controls. Control, thermally induced release (% of acid phosphatase and β-glucuronidase in vehicle-treated rats was 35.3 ± 2.3 (mean ± SEM) and 40.3 ± 2.2, respectively (N = 50), of hypotonically induced release.

4 Concentrations of vitamin E up to 7.8 IU/100 ml or Se up to 16.0 /µg/100 ml had no effect, in vitro, on free lysosomal enzymes obtained from hypotonically disrupted lysosomes, when incubated in 0.25 M sucrose for 90 minutes at 45°.

5 Plus signs indicate augmented release of enzymes, compared to control, thermally induced release.

of 0.2 M glycine buffer, pH 10.4; OD was measured at 540 mµ. Control tubes contained the same mixture except that 0.05 or 0.15 of distilled water replaced the AP or BG solutions, respectively.

To ascertain whether the compounds to be studied had any direct effects on lysosomal enzyme activity per se, half of the lysosome-rich fraction was suspended in distilled water to half of the usual volume (7.5 ml), incubated at 22° for 30 minutes and centrifuged (25,000 × g, 4°, 15 minutes). To 1.5 ml of the remaining “free” enzyme solution, 1.5 ml of drug or sucrose solution was added, and the tubes were incubated for 90 minutes at 45°. Triamcinolone acetonide solutions contained a final concentration of 10⁻⁴ or 10⁻⁴ M in 0.25 M sucrose, vitamin E solutions were 7.80 or 0.78 IU/100 ml, and selenium solutions contained 16 or 1.6 /µg/100 ml selenium equivalent. A water-soluble E (d-a-tocopheryl polyethylene glycol 1000 succinate *) was used in these in vitro studies.

RESULTS

Initial experiments were conducted to determine whether substrate concentrations used in the enzyme assays were sufficient for the amounts of enzymes released thermally or hypotonically. Absorbance values (used to express enzymic activity) were directly proportional to the volume (amount) of free lysosomal enzymes added; the volumes of enzyme solutions used for assaying acid phosphatase and β-glucuronidase (0.05 ml and 0.15 ml, respectively) were located within the linear portions of these curves.

The degree of stabilization of lysosomes by vitamin E increased with increasing dosage (table 1). Maximal stabilization was attained with 0.100 unit/kilogram. Stabilization of lysosomal membranes is indicated by the attenuated release of both acid phosphatase and β-glucuronidase. That both of these enzymes were derived from the same source is indicated by the almost identical degree of thermally induced release, and by the almost identical degree of inhibition of release (tables 1, 2). Such results also strongly suggest a lack of interference with release of enzymes or enzyme activity per se by any mitochondria or microsomes which may have been present. Selenium at 2 /µg/kg also stabilized lysosomes, but at higher or lower dose levels this effect was not ob-
TABLE 2
Attenuation of thermally induced (45°) release of lysosomal enzymes from hepatic lysosomes by steroidal antiinflammatory drugs

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Daily dose (µg/kg ip)</th>
<th>N</th>
<th>Inhibition (%) of enzyme release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AP</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>25</td>
<td>4</td>
<td>31 *</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4</td>
<td>54 *</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4</td>
<td>45 *</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>200</td>
<td>4</td>
<td>41 *</td>
</tr>
<tr>
<td>Acetonide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>100</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>3</td>
<td>13 *</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7</td>
<td>40 *</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>4</td>
<td>59 *</td>
</tr>
</tbody>
</table>

*AP = acid phosphatase; BG = β-glucuronidase.

Results demonstrate a suitable assay system for studying the effects of lysosomal-stabilizing agents in vivo on lysosomes isolated after administering such agents to rats for 3 days. Substrate concentrations selected for enzyme determinations in this system were sufficient for the amounts of enzymes released thermally or hypotonically. As a result of preliminary experiments, isolated hepatic lysosomes were incubated in 0.25 M sucrose only. Exogenous buffer was not necessary since the pH of the resulting lysosomal suspension remained 6.8 due to endogenous buffer systems. Moreover, the administration of drugs in vivo eliminates the need of exogenous buffering during incubation since no drug has to be added directly, thus avoiding a possible correction factor due to direct enzyme inhibition, although the latter should nonetheless be determined.

In the experiments reported herein animals received a commercial stock diet (65 IU/kg vitamin E) ad libitum. Each consumed an average of 20 g of food per day, in agreement with data of Goyer et al. (16), which is equivalent to 1.3 IU/rat/day. Absorption of vitamin E, however, is quite low (17). Goyer et al. (16) found that rat liver contains 15 µg of vitamin E per gram of liver (for livers weighing 6.2
but only 63 µg of vitamin E when total vitamin E was expressed per 100 g body weight for rats weighing about 125 g. Since their rats consumed 370 µg of vitamin E per day, and the liver contained more vitamin E than other tissues as is obvious from the data above or from experiments of Mason (17), either the absorption of vitamin E is quite low or the metabolism of vitamin E is quite high. An increased intake of vitamin E may thus be expected to augment the content of vitamin E in the liver, especially if the vitamin is injected intraperitoneally. After intraperitoneal absorption, substances are carried via the portal vein to the liver wherein it is well known that a large portion of injected drugs, and probably vitamin E (17), is extracted from the blood during the first circulatory transit through the portal vein–sinusoid–hepatic vein circuit. Absorption of drugs after oral administration takes a similar course. Concentration of vitamin E in the liver was described by Mason (17). In that study it was shown that the liver of rats can concentrate vitamin E as much as four to fourteen times, depending upon whether there was a low or high level of intake of vitamin E. Thus, in the experiments reported herein, one could consider the effect of vitamin E to be either a nutritional effect \(^1\) or a pharmacologic effect. Our daily dosages of 0.039 to 3.9 IU/kg of vitamin E are also well within the range of 0.8 to 1.2 mg (1.2 to 1.8 IU) of \(\alpha\)-tocopherol (iv) per normal rat required to inhibit hemolysis induced by hydrogen peroxide, 24 to 48 hours after the rats received vitamin E (18). Since the selenium content of the commercial diet is not determined by the manufacturer and therefore is unknown, it is certainly possible that the diet could have been marginally deficient in selenium. If so, the results with exogenous vitamin E or selenium must be interpreted as nutritional effects.

Inhibition of release of lysosomal enzymes by dexamethasone was observed at dose levels that are equivalent to the usual pharmacologic dosages administered to man (0.75 mg, once to three times per day). Such dose levels in a 70-kg man are 10 to 33 µg/kg. Since dexamethasone is 25 times more potent than cortisol (19), such a dosage is equivalent to 18 to 58 mg cortisol per 70-kg man. This dosage is well within the range of normal, daily secretion of cortisol by the adrenal cortex of a normal human subject (19). On this basis one would probably not expect a therapeutic effect, yet dexamethasone is very effective in many clinical situations at this dosage; indeed, this or higher dosage causes severe side effects (19, 20). Moreover, 5 to 10 µg/kg of dexamethasone is effective in attenuating polyarthritis in rats after oral (21) or intraperitoneal administration (unpublished results). At ten times higher dosages rats do not survive. This dosage is also slightly less than that reported herein to diminish the thermally induced release of lysosomal enzymes. Thus the liver is probably also concentrating dexamethasone as it does vitamin E. There may also be a narrow margin between efficacy and side effects with vitamin E, as noted above in rats for dexamethasone or as described by Sones and Slocumb (20). Thus, at the 3.9 IU/kg dosage of vitamin E (table 1) release of only one of the lysosomal enzymes is significantly inhibited. Still larger amounts of vitamin E (0.5 ml) have been employed to induce inflammation in air pouches on the dorsum of rats (22).

Nutritionally, vitamin E may be considered by some as an antisterility factor, but from the standpoint of pharmacodynamics it seems more pertinent to describe vitamin E as a nutritional, antiinflammatory factor. It thus seems more than coincidental that either vitamin E or antiinflammatory drugs stabilize erythrocytes to hemolysis (23–27) induced by thermal stress or hydrogen peroxide. Likewise, it seems more than coincidental that antiinflammatory drugs stabilize capillaries or venules of the microvasculature to various agonists, thereby blocking edema and swelling as does vitamin E in preventing the increase in capillary permeability resulting in edema and swelling associated with myocardial lesions, nutritional encephalomalacia, and exudative diathesis (17). One of the principal metabolites of vitamin E (tocopheronolactone) considered by some investigators (28) to be the

\(^{1}\) Provided the commercial stock diet was marginally deficient in vitamin E.
active metabolite of vitamin E, does possess antiinflammatory activity in pharmacologic assays and also inhibits the release of acid phosphatase and \( \beta \)-glucuronidase in vivo or in vitro (29). Moreover, stabilization of lysosomes by vitamin E could explain prevention of dietary necrotic liver degeneration, since release of various proteolytic enzymes contained therein would be prevented. Steroidal or nonsteroidal antiinflammatory drugs have not been tested in the latter manifestation of vitamin E deficiency but may prove to be effective. Furthermore, supplementation of vitamin E-deficient diets with vitamin E attenuates kidney autolysis in rats and also partially protects the kidney lysosomes against increased fragility (30).

Whether the membrane-stabilizing effect of selenium, vitamin E or steroidal antiinflammatory drugs can be reproduced on lysosomes of rat liver obtained from animals fed diets deficient in vitamin E or selenium is speculative. Nevertheless, the data reported herein suggest that such an effect is feasible. Buchanan-Smith et al. (10) studied the lability of hepatic lysosomes of sheep on a basal diet containing selenium, vitamin E or both. Since no control animals (on normal diets or basal diets only) were included in this study (because of death in animals on basal diets only), the effects of these substances on lysosomal stability in comparison to the stability of hepatic lysosomes derived from normal animals, or animals on a basal diet per se, are unknown. It is known, however, that small amounts of vitamin E added in vitro to hepatic lysosomes derived from vitamin E-deficient rats will decrease the lability of such lysosomes (11). Since severe selenium deficiency impairs absorption of vitamin E (6), selenium may potentiate vitamin E by increasing plasma levels of tocopherol. Such an interaction, however, does not fully explain the ability of either selenium or vitamin E to prevent exudative diathesis in chicks (6) or hepatic necrosis in rats (1). Low dose levels of selenium (0.5 \( \mu \)g/kg) potentiated vitamin E (0.039 unit/kg), whereas high dosage of selenium (50 \( \mu \)g/kg) antagonized the stabilizing effect (table 1) of vitamin E at a dosage of 3.4 units/kg, i.e., this combination results in significant (\( P < 0.05 \)) labilization of the lysosomal membranes. Thus, the potentiating effects of these two substances in exudative diathesis or muscular dystrophies may be attributed to a positive interaction in stabilizing lysosomes, since this was observed.

**LITERATURE CITED**


