Selenoenzymes Regulate the Activity of Leukocyte 5-Lipoxygenase via the Peroxide Tone*

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The variation of the selenium status of leukocytes was used as a tool to investigate the influence of selenium-containing glutathione peroxidases on the formation of 5-lipoxygenase metabolites in vitro and ex vivo. Selenium-deficient rat basophilic leukemia cells had <1% of control glutathione peroxidase activity and 35% of control phospholipid hydroperoxide-glutathione peroxidase activity. Upon stimulation, these cells released an 8-fold amount of lipoxygenase metabolites compared to controls. No (5S)-hydroperoxyeicosatetraenoic acid was detectable in whole cells; however, it was found in homogenates of selenium-deficient cells.

Addition of 0.25 μg/ml selenium to selenium-deficient cells restored control phospholipid hydroperoxide-glutathione peroxidase activity within 6 h, whereas glutathione peroxidase activity needed 7 days. 12 h after resupplementation, selenium-deficient cells had 3% glutathione peroxidase and 100% phospholipid hydroperoxide-glutathione peroxidase activity compared to controls. Resupplemented cells released control amounts of 5-lipoxygenase metabolites, indicating that restoration of phospholipid hydroperoxide-glutathione peroxidase activity is associated with a selenium-adequate leukotriene metabolism.

Leukocytes that were isolated from selenium-deficient rats released a 7-fold amount of total lipoxygenase metabolites compared to cells from control animals. By injecting normally fed rats with 500 μg/kg selenium as Na₂SeO₃, leukocyte phospholipid hydroperoxide-glutathione peroxidase activity was raised 8-fold within 114 h compared to controls. Leukocytes from these animals produced significantly less lipoxygenase metabolites than controls.

These findings indicate that phospholipid hydroperoxide-glutathione peroxidase activity is primarily responsible for the reduction of 5-hydroperoxyeicosatetraenoic acid and therefore governs the actual activity of leukocyte 5-lipoxygenase via regulating the tone of endogenous hydroperoxides.

Selenium is an essential trace element for animals and man. Deficiency of this element causes a variety of symptoms in different mammalian species that are hard to reconcile with a single biochemical function. Children with a low selenium status develop a cardiomyopathy (Keshan disease) characterized by chronic inflammation of the joints, degradation of cartilage, and impairment of function. The latter condition is improved by selenium supplementation (2); however, a causal involvement of selenium deficiency in this disease awaits proof. A possible molecular basis of these selenium-responsive diseases is the action of hydroperoxides as potent endogenous proinflammatory stimuli. In healthy man, the steady-state levels of these hydroperoxides are assumed to be influenced by at least two different selenium-containing enzymes, namely the so-called "classical" glutathione peroxidase as well as the phospholipid hydroperoxide-glutathione peroxidase. Glutathione peroxidase is a soluble protein containing 4 g atoms of selenium/tetramer, is highly specific for its donor substrate (GSH), and reduces a wide variety of chemically different hydroperoxides including H₂O₂, t-buty1 hydroperoxide, cumene hydroperoxide, and fatty acid hydroperoxides (3). In contrast, phospholipid hydroperoxide-glutathione peroxidase, containing 1 g atom of selenium/single molecule (5), acts only poorly on H₂O₂, but reduces fatty acid hydroperoxides and preferentially phospholipid hydroperoxides including cholesterol hydroperoxide (6). During dietary selenium deficiency in mice, glutathione peroxidase activity of major organs is rapidly lost, whereas phospholipid hydroperoxide-glutathione peroxidase activity is only partially decreased even after months (7).

Hydroperoxides relevant to inflammation are those formed from arachidonic acid by stereoselective lipoxygenases that need the fatty acid substrate to be released from phospholipid via phospholipase A₂. The stereoselective enzyme 5-lipoxygenase requires high-molecular cytosolic and membrane-bound proteins, calcium ions, and ATP as well as a low hydroperoxide concentration for its full activation. Its catalytic cycle includes a translocation of the catalytic subunit from the membrane to the vicinity of a protein named 5-lipoxygenase-activating protein (9). Thus, the formation of the primary lipoxygenase product, i.e. 5-HPETE, takes place at the membrane in close association with phospholipase A₂, 5-lipoxygenase-activating protein, and 5-lipoxygenase itself. An increased steady state of HPETE activates 5-lipoxygenase up to concentrations of ∼20 μmol/liter via the so-called peroxide tone (10). At higher concentrations, an oxidative self-inactivation of the enzyme becomes prevalent (11). The rate of reduction of 5-HPETE to 5-HETE determines the synthesis of leukotriene A₄, the common precursor of the different leukotrienes. Since 5-HPETE controls its own synthesis, the rate of HPETE reduction becomes the critical determinant of leukotriene synthesis under physiological conditions. It is

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1 The abbreviations used are: HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LT₄, leukotriene B₄, HPLC, high pressure liquid chromatography.

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assumed that selenium-containing GSH peroxidases are potential candidates for the metabolism of the autoregulator HPETE.

We therefore used a rat leukocyte cell line capable of synthesizing leukotrienes as an in vitro system in which the activity of these enzymes was modulated above and below their physiological levels by manipulation of the selenium status of these cells. In a second approach, the validity of the results obtained in the cell culture system was checked ex vivo in leukocytes from selenium-deficient and control rats and from rats with an artificially raised selenium status.

**MATERIALS AND METHODS**

**Substrates and Enzyme Assays**—The substrate for phospholipid hydroperoxide glutathione, i.e. phosphatidylcholyl hydroperoxide, was synthesized as described previously (7). Phospholipid hydroperoxide-glutathione peroxidase activity was assayed according to Ref. 7 as follows. 10 μl of biological sample were added to a total volume of 1.0 ml containing 0.1 mol/liter Tris, pH 7.6, 5 mmol/liter EDTA, 1 mmol/liter azide, 3 mmol/liter GSH (Sigma), 0.1% peroxide-free Triton X-100 (Boehringer Mannheim), 0.1 mmol/liter NADPH (Boehringer Mannheim), and 1.2 units of glutathione reductase (Boehringer Mannheim; specific activity of 120 units/mg of protein). The reaction was started at 37 °C by addition of 10 μl of phospholipid hydroperoxide in methanol (final concentration of 57 μmol/liter), and the change in absorbance at 340 nm was recorded (Kontron Uvikon 930 spectrophotometer). (5R,10R)-3-[(1-hydroxy-2-phenyl)-5-isopropyl-3-tert-butylthiocolindol-2-yl]-2,2-dimethylpropanoic acid was a gift of Merck Frosst Canada, Inc. (Montreal). Eicosatetraenoic acid and norlidihoxyuguaiceric acid were from Sigma. Ebselen (2-phenyl-1,2-benziselenazol-3(2H)-one) was from Rhône-Poulenc Rorer Nattermann International (Köln, Germany).

Glutathione peroxidase was assayed with H2O2 (Merck, Darmstadt, Germany) as described (12). Protein was measured according to the procedure of Bradford (13).

**Reversed-phase HPLC Analysis**—The separation of 5-lipoxygenase products (65)-hydroxyeicosatetraenoic acid, (5S)-hydroxyeicosatetraenoic acid, and LTB4, and its isomers) was performed using an HPLC system with a reversed-phase column (5-μm ODS-Nucleosil C18, 250 x 4.6 mm) as described (14). The amount of 5-lipoxygenase products was determined by UV measurement (wavelength of 237 nm for conjugated dienes ((SS)-HETE/(SS)-HPETE)) and of conjugated trienes ((LTB4,)). Quantification was carried out using the molar extinction coefficients of the different metabolites for a calibration curve. As internal extraction standard, 50 pmol of phosphatidylcholyl hydroperoxide in methanol (final concentration of 57 μmol/liter) and of peroxide-free Triton X-100 (0.1 mmol/liter) was added to each incubation.

**Ex Vivo Experiments**—Male Wistar rats (Dr. Karl Thomae, Chemische- pharmaceutische Fabrik, Biberach, Germany) were maintained in Macrolon cages at 55% humidity and 22 °C with an artificial 12-h day/night rhythm. The animals were fed for 6 weeks from weaning a diet according to Ref. 15, which contained <1 ppm selenium. Controls received an identical diet supplemented with 0.5 ppm selenium in the form of Na2SeO3. Alternately, the animals were supplemented by intraperitoneal injection of 0.5 mg/kg selenium in the form of Na2SeO3 in 0.9% NaCl 1 h before isolation of the granulocytes. Controls received the same volume of saline.

To extract a maximum blood volume, the animals were anesthetized by intraperitoneal injection of 60 mg/kg Nembutal (Sanofi-Winthrop, GmbH, München, Germany). Blood was taken from the vena pulsionalis after opening of the thorax. Coagulation was prevented by intracutaneous injection of 150 units of heparin/ml of blood.

The preparation of polymorphonuclear granulocytes from the blood of rats was performed by subsequent centrifugation on Percoll as described (16). The viability as measured by trypan blue exclusion was >97%. With Wright’s stain, the cell suspensions were estimated to contain an average of 96–98% neutrophils. After isolation, the cells were stored at 4 °C in NaCl/phosphate (137 mmol/liter NaCl, 2.7 mmol/liter KCl, 8.1 mmol/liter NaHPO4, 1.5 mmol/liter KHP04, pH 7.4, 310 mOsm/liter).

**Cell Culture Experiments**—Rat basophilic leukemia (RBL-1) cells were cultured for 5 days on a selenium-deficient medium or, alternatively, on a medium containing 0.25 ppm selenium in the form of Na2SeO3. Under standard conditions, we used Dulbecco’s modified Eagle’s medium with 3.7 g/liter NaHCO3, 4.5 g/liter d-glucose, pH 7.5, with 10 kiloiunits/ml penicillin, 10 mg/ml streptomycin, 0.2 mol/liter t-glutamine, 0.1 mol/liter sodium pyruvate, 0.25 mg/ml amphotericin B, 0.75% fetal calf serum, and 9.25% basal medium supplement. Under these conditions, no differences in the growth rates (doubling time of 24 h) of selenium-deficient and selenium-supplemented cells were observed. For incubation, cells were harvested by centrifugation (10 min, 400 x g, 4 °C) and washed twice with NaCl/phosphate. The viability of the cells was measured by trypan blue exclusion and was >96%.

Cell suspensions (5 x 106 cells/ml) in NaCl/phosphate were sonicated at 4 °C (Branson Sonic Power Co., cell disruptor B-30, 20 pulses at the microtip limit). The homogenate was centrifuged for 10 min at 10,000 x g and 4 °C, and the supernatant was used for the experiments.

Cells or the 10,000 x g supernatant of leukocytes were preincubated with 1 mm CaCl2 at 37 °C. 5-Lipoxygenase activity was stimulated by addition of 5 μmol/liter exogenous arachidonic acid and 2 μmol/liter ionomycin in the case of RBL-1 cells and of 50 μmol/liter exogenous arachidonic acid and 2 μmol/liter ionomycin in the case of rat polymorphonuclear granulocytes. Ionomycin was omitted in the homogenates. The incubation was terminated after 5 min by extraction into acetic ester at pH 3.0, followed by HPLC analysis of the 5-lipoxygenase products.

**RESULTS**

The first objective of this study was to determine conditions under which RBL-1 cells can be cultivated under selenium deficiency without loss of basic functions such as viability, growth rate, and adhesion properties as well as microscopic appearance. Since the fetal calf serum as a nonsynthetic ingredient (in contrast to the fetal medium used by us) contained significant amounts of selenium, a series of culture experiments was aimed at reducing the fetal calf serum content until selenium deficiency was reached without loss of viability. The data in Table I show that 0.7% fetal calf serum was a condition under which cellular selenium was severely depleted after 5 days of culture. Supplementation of these selenium-deficient cells with 0.25 ppm selenium was chosen for control conditions because this is a physiological (7) and noncytotoxic supplementation range. Selenium-deficient cells had 100 times less glutathione peroxidase activity and 2.5 times less phospholipid hydroperoxide-glutathione peroxidase activity than control cells (Table I). This responsiveness of RBL-1 cell GSH peroxidases to selenium deficiency matches well the conditions in whole animals (7).

The experiments were performed on a time course of selenium repletion by Na2SeO3 of selenium-deficient RBL-1 cells. The results demonstrate that sodium selenite is not only taken up into the cells (Table I).

**TABLE 1**

Selenium and selenoenzyme status of RBL-1 cells cultured under control and selenium-deficient conditions

| Parameter | Control | Se-deficient |
|-----------|---------|-------------|
| Selenium content (ng/ml) | 149.5 | 4.2 |
| GPX* activity (millimunits/ml) | 28.0 ± 1.1 | 2.6 ± 0.03 |
| PH-GPx activity (millimunits/ml) | 5.2 ± 0.2 | 2.1 ± 0.1 |

*GPX, glutathione peroxidase; PH-GPx, phospholipid hydroperoxide-glutathione peroxidase.
but is also incorporated into selenoenzymes. The results illustrate that phospholipid hydroperoxide-glutathione peroxidase activity increased very rapidly in selenium-deficient cells from 30% to control activity within the first 8 h. At this time, glutathione peroxidase activity was still as low as 3% of control and needed almost 7 days to reach control levels. This completely different time course of the two selenoenzymes therefore allows us to check the contribution of either activity by setting an experiment at a time point later than 8 h after resupplementation.

In the following series of experiments, selenium-deficient and control RBL-1 cells were stimulated to form lipoxygenase metabolites under various conditions. When no exogenous arachidonic acid was added to the cellular system, no lipoxygenase metabolites were detected in either status. Therefore, we decided to stimulate the cells with exogenous arachidonic acid. A concentration of 10 μmol/liter was optimal for this purpose without showing cytotoxicity as judged by the trypan blue exclusion method. Over a time scale from 30 s to 20 min, the maximum rate of lipoxygenase metabolite formation occurred around 5 min. Lipoxygenase metabolite formation was fully inhibited independent of the selenium status in the presence of the following lipoxygenase inhibitors: 10 μM nordihydroguaiaretic acid, 50 μM eicosatetraynoic acid, 0.1 μM MK-886, or 30 μM Ebselen.

Under these conditions of substrate supply and stimulation, the role of the selenium status with respect to lipoxygenase activity can be stimulated in RBL-1 cells and that the actual activity depends on their selenium status.

To study whether selenium deficiency induces or stimulates lipoxygenase activity, we repeated the same type of experiments in a cell-free system. The data in Table II show that under control conditions, cell homogenates from RBL-1 cells produced much more lipoxygenase metabolites than whole cells. Moreover, HPETEs were also detected in homogenates.

### Table II

| Selenium status | Whole cell | Cell-free |
|-----------------|------------|-----------|
|                 | Control    | Se-deficient | Control    | Se-deficient |
| LTB₄            | 2.1 ± 0.5  | 44 ± 2     | 46 ± 2     | 34 ± 0.9    |
| (5S)-HETE       | 41 ± 6     | 332 ± 15   | 244 ± 15   | 140 ± 21    |
| (5S)-HPETE      | ND         | ND         | 83 ± 10    | 120 ± 33    |
| Total amount of 5-lipoxygenase metabolites | 43 ± 4.3 | 375 ± 15 | 372 ± 15 | 294 ± 13 |

In contrast to the whole cell system, selenium deficiency did not increase lipoxygenase metabolite formation. Therefore, it seems that under these conditions, a further increase of lipoxygenase activity by selenium-dependent processes does not become manifest.

The detectability of HPETEs in selenium-deficient and control homogenates may be due to lack of a sufficient amount of substrate for the peroxidases, i.e., GSH, due to the dilution of the supernatant during preparation. To check this possibility, 1 mmol/liter GSH was added to selenium-deficient or control RBL-1 homogenates, and lipoxygenase metabolites were then measured. The data in Table III show that in the presence of GSH in homogenates from selenium-deficient cells, HPETE was still found, whereas it was no longer detectable in control homogenates with exogenously added GSH. This experiment demonstrates that a selenium-dependent process needing GSH reduces HPETE.

In the second part of this study, the relevance of these in vitro data for the in vivo situation was examined. Therefore, granulocytes were prepared from rats kept on a selenium-deficient diet for 6 weeks and from control animals fed either a normal laboratory diet or a selenium-supplemented control diet. The granulocytes from selenium-deficient animals had a glutathione peroxidase activity of <3% of controls and a phospholipid hydroperoxide-glutathione peroxidase activity of 35% of controls (Table IV). These enzyme data show that ex vivo leukocytes were depleted to an extent and ratio similar to those of the RBL-1 cells in culture. No difference was found in the selenium status between the animals fed the standard diet and the animals fed the selenium-supplemented control diet (Table IV). This demonstrates that the supplementation in the control diet was adequate. Cells from selenium-deficient animals produced ~7-fold more lipoxygenase metabolites than the two control cell groups (Table IV),

### Table III

| Control cells | Se-deficient cells |
|---------------|--------------------|
| ng            | ng                 |
| No addition   | 108                | 182               |
| + 1 mmol/liter GSH | 2.2                | 156               |
TABLE IV
Selenoenzyme activities and ex vivo 5-lipoxygenase product formation in granulocytes from selenium-deficient, selenium-supplemented (control), or normally fed male Wistar rats

|                     | Normal diet | Control | Se-deficient |
|---------------------|-------------|---------|-------------|
| GPx activity (milli-units/mg) | 586         | 570     | 15.5        |
| PH-GPx activity (milli-units/mg) | 0.7         | 0.8     | 0.25        |
| 5-Lipoxygenase product formation (ng) | 44          | 45      | 298         |

demonstrating in vitro and ex vivo analogy.

In another independent experiment, leukocytes from selenium-deficient rats were stimulated with 1 mmol/liter CaCl₂ and 5 μmol/liter ionophore without addition of exogenous arachidonic acid. A significant increase of the 5-lipoxygenase metabolites measured (LTB₄, (5S)-HETE, 6-trans-LTB₄, and 6-trans-12-epi-LTB₄) of 28 ± 13.6% (p < 0.01, double-sided, untailed t test) was found in selenium-deficient cells. This shows that the basic effect induced by exogenous arachidonic acid is also present using a physiological stimulus in cells endowed with endogenous phospholipase A₂ activity.

Another experiment was required to distinguish the individual contribution of each peroxidase. When rats fed a standard diet were injected with 500 μg/kg selenium, their phospholipid hydroperoxide-glutathione peroxidase activity in granulocytes increased 8-fold after 114 h, whereas glutathione peroxidase activity increased only 1.6-fold. If the enhanced lipoxygenase activity observed in selenium-deficient rats is the result of an enhanced hydroperoxide tone, then the artificial elevation of the responsible enzyme, i.e., phospholipid hydroperoxide-glutathione peroxidase, should decrease the lipoxygenase metabolite formation under these conditions. Actually, granulocytes from selenium-injected rats with 8-fold phospholipid hydroperoxide-glutathione peroxidase activity produced only 100 ± 36 (n = 7) ng of lipoxygenase metabolites/5 × 10⁶ cells compared to 300 ± 100 ng (n = 3) from normally fed rats. This experiment extends and corroborates our previous conclusion that selenium-dependent phospholipid hydroperoxide-glutathione peroxidase governs the hydroperoxide tone in leukocytes by reducing HPETEs and therefore ultimately regulates the actual lipoxygenase activity.

DISCUSSION

In previous reports, several pieces of circumstantial evidence were obtained that GSH peroxidases can affect the hydroperoxide tone of white blood cells and thereby influence the lipoxygenase activity. Addition of purified bovine glutathione peroxidase to homogenates from RBL-1 cells was reported to result in inhibition of product formation of lipoxygenase (17). In human granulocytes, it was demonstrated that depletion of cellular GSH led to an increase in 5-lipoxygenase products and that this effect is most pronounced below a threshold of 20 μmol/liter arachidonic acid (18). Moreover, in platelets from selenium-deficient rats, an increased 12-lipoxygenase activity was found (19). These findings suggested an interrelation between peroxide tone and a GSH-dependent reaction requiring selenium.

In this study, selenium deprivation and restoration combined with the measurements of the two GSH peroxidases were used as a direct approach to demonstrate the regulatory role of these enzymes in leukotriene formation. The metabolism of arachidonic acid requires its receptor-stimulated release from phospholipids under physiological conditions. In our RBL-1 cells, arachidonic acid was incorporated into the phospholipids, but no metabolite formation could be induced, indicating that phospholipase A₂ is not functionally active in these cells. Therefore, exogenous arachidonic acid was added as a substrate of 5-lipoxygenase, a situation that resembles the cooperation of different leukocyte populations in providing precursors of leukotriene synthesis. The inhibition of the formation of any product of 5-lipoxygenase measured by chemically and mechanistically different inhibitors of this stereoselective white blood cell enzyme activity excludes a nonenzymatic source of the metabolites detected. The activities of 12- or 15-lipoxygenases in the RBL-1 cells were smaller than 5% of the 5-lipoxygenase activity and therefore were not further investigated.

The ex vivo experiments with granulocytes from selenium-deficient rats demonstrate that increased 5-lipoxygenase product formation in selenium-deficient cells is also observed after activating the release of endogenous arachidonic acid with calcium and ionophore. The fact that this increase under endogenous stimulation was not as large as that with exogenous arachidonic acid may be ascribed to a different utilization of endogenous arachidonic acid in the re-acylation pathway compared to arachidonic acid passing a gradient over the cell membrane from outside.

In the whole cell system, significant amounts of HPETEs were detected only within the first minute under selenium-deficient conditions. This shows on the one hand that, at least transiently, an enhanced peroxide tone is produced in the cells. On the other hand, the residual activity of phospholipid hydroperoxide-glutathione peroxidase may be sufficient to reduce these HPETEs as long as intracellular GSH is available. Only in selenium-deficient homogenates did PHETE accumulate even in the presence of GSH. This means that in control homogenates under artificial conditions, the cytosolic glutathione peroxidase reduces free fatty acid hydroperoxides. The disintegration of the close association of the regulatory proteins to which the catalytic lipoxygenase subunit is translocated may explain why these homogenates show no difference between selenium-deficient status and control status was found. Therefore, within the whole cell under physiological conditions, a regulation via the peroxide tone is likely to operate directly in the membrane without release of a hydroperoxide to a compartment where it would be accessible to glutathione peroxidase.

For the ex vivo experiments, it was important to work at a physiological selenium supplementation range. Since no differences in selenoenzyme activities were detected between the supplemented groups and the animals fed a normal diet, it can be assumed that the amount and chemical form of selenium were as adequate as those in the standard diet. However, remarkable differences were seen when different routes of selenium supply were used. In comparison to the dietary supplementation, intraperitoneal administration of selenium preferentially increased the leukocyte phospholipid hydroperoxide-glutathione peroxidase activity. The reason for this phenomenon is unknown. However, it offers a way of studying the individual contributions of either enzyme activity to the regulation of the peroxide tone. The fact that only a change in phospholipid hydroperoxide-glutathione peroxidase activity had consequences on lipoxygenase metabolite production rates corroborates the view that phospholipid hydroper-
oxido-glutathione peroxidase is the enzyme responsible for this process.

Also, previous literature supports the interpretation that phospholipid hydroperoxide-glutathione peroxidase is the primary enzyme for reduction of endogenous HPETE. In human granulocytes, it was observed that the enzyme activity that reduces HPETE to HETE cannot be inhibited by mercaptosuccinate (20). It was concluded that glutathione peroxidase, which is inhibitable by mercaptosuccinate (21), cannot account for this reaction. In our hands, phospholipid hydroperoxide-glutathione peroxidase activity in mouse organ or white blood cell homogenates was not inhibitable by mercaptosuccinate. We found also that phospholipid hydroperoxide-glutathione peroxidase is not strictly specific for GSH and uses different thiols such as mercaptoethanol and diethithreitol. These findings also provide an interpretation for the observation that 12-HPETE reduction in thrombocytes could be achieved not only by GSH, but also by other thiols (19).

The results presented in this study may have a bearing on chronic selenium deficiency conditions in man such as Kashin-Bek disease. They suggest an increased tendency of the release of inflammatory mediators under extremely deficient conditions. This does not necessarily mean that below saturation with the element within physiological variation of selenium supplementation, a proinflammatory metabolism has to be expected. On the contrary, the experiments summarized in Fig. 1 and Table IV demonstrate that marginal amounts of selenium are sufficient to normalize leukotriene metabolism.

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