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Isolation and Characterization of Deteriosomes From Rat Liver

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Abstract Deteriosomes, a new class of microvesicles, have been isolated from rat liver tissue. These microvesicles are similar to those isolated previously from plant tissue [Yao et al., Proc Natl Acad Sci USA 88:2269–2273, 1991] in that they are nonsedimentable and enriched in membrane catabolites, particularly products of phospholipid degradation. Liver deteriosomes range in size from 0.05 μm to 0.11 μm in radius. They are also much more permeable than microsomal membrane vesicles indicating that the deteriosome bilayer is perturbed. The data are consistent with the proposal that deteriosomes are formed from membranes by microvesiculation and that they represent an intermediate stage of membrane deterioration. Furthermore, liver deteriosomes were found to contain phospholipase A_2 activity. This suggests that they not only serve as a means of moving destabilizing macromolecular catabolites out of membranes into the cytosol but also possess enzymatic activity. The fact that the specific activity of phospholipase A_2 is higher in deteriosomes than in deteriosome-free cytosol suggests that some of the enzymatic activity traditionally assumed to be cytosolic may in fact be associated with deteriosomes.

Key words: membrane breakdown, permeability, nonsedimentable microvesicles, phospholipase A_2, calcium

Phospholipid degradation, which is facilitated by membrane-bound phospholipase activities, plays an important role in membrane lipid turnover. In animal systems, phospholipase A_2 activity, which cleaves fatty acids from the *sn*-2 position of phospholipids, has been detected in isolated plasma membranes, microsomal membranes, and zymogen granule membranes [22,23,28]. Phospholipase A_1 activity has also been found associated with plasma membranes and microsomal membranes [21,23]. This enzyme releases fatty acids from the *sn*-1 position of the phospholipid glycerol backbone. There are also reports of membrane-associated phospholipase C, which generates diacylglycerols from phospholipids [7,9,10,17,26]. The collective activities of these membrane-bound phospholipases generate free fatty acids, lysophospholipids, and diacylglycerols. Any significant accumulation of phospholipid degradation products in membranes could perturb the membrane bilayer. For example, accumulation of saturated free fatty acids forms gel phase domains in membrane bilayers [14]. The coexisting liquid-crystalline and gel phase lipid domains render the membrane bilayer leaky [3]. Also, some phospholipid degradation products are fusogenic, the accumulation of which promotes membrane fusion and microvesiculation. For example, it has been reported that diacylglycerols induce membrane microvesiculation from erythrocytes and phospholipid bilayers [1,2,8] and that free oleic acid enhances the fusion of phospholipid bilayers [15,18].

It has been established recently in plant systems that phospholipid degradation products are removed from membranes by microvesiculation to form deteriosomes (nonsedimentable microvesicles) [30,31]. Deteriosomes are enriched in membrane catabolites, especially products of phospholipid degradation, which may contribute to their nonsedimentable nature [31]. It has been proposed that plant deteriosomes function as an intermediate stage of membrane deterioration. In the present study, we have isolated deteriosomes from rat liver tissue. The chemical properties and permeability of these microvesicles were characterized and compared with microsomal membranes. Also, the activity of phospholipase A_2 was assayed to determine if animal deteriosomes are enzymatically active.

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MATERIALS AND METHODS

Isolation of Microsomal Membranes and Deteriosomes

The isolation of microsomal membranes and deteriosomes was based on procedures described previously [31]. Briefly, livers, excised immediately after rats were killed, were minced with scissors and homogenized in a teflon-glass homogenizer at 500 rpm for 1 min in buffer of 50 mM $NaHCO_3$–300 mM sucrose, pH 7.0 at 4°C. The homogenate was centrifuged at 10,000*g* for 20 min, and the pellet was discarded. The supernatant was centrifuged at 130,000*g* for 1 h to yield microsomal membranes. The post-microsomal supernatant was centrifuged at 250,000*g* for 12 h to sediment any residual membrane and to float residual triacylglycerol (which was removed prior to ultrafiltration), and the pellet, which contains residual membranes, was discarded. The resulting supernatant, which contains deteriosomes, was concentrated from 50 ml to 10 ml by filtration through a 1,000 kDa Pharmacia cut-off filter. The filtrate (deteriosome-free cytosol) was collected for further analysis. The concentrated deteriosomes, which did not pass through the filter, were washed three times with 2 volumes of isolation buffer to remove residual cytosol and then used for subsequent characterization studies. The washing procedure reduced contamination by cytosolic protein to $< 0.7\%$.

Compositional Characterization of Deteriosomes

Lipids from microsomal membranes and deteriosomes were extracted and analyzed by thin-layer chromatography (TLC) as described previously [12,31]. Total lipid extracts were spotted onto silica gel 60 plates (Mandel, Guelph, Ontario). The plates were developed halfway in chloroform/acetic acid/methanol/water (70:25:5:2; v/v), dried under N_2, and then completely developed in petroleum ether/diethylether/acetic acid (70:30:1; v/v). Proteins were quantified according to Bradford [5], separated by SDS-PAGE on 8–16% linear gradients as described by Laemmli [16], and stained with silver [27].

Size Distribution by Dynamic Light Scattering

Isolated deteriosomes (100 μg protein/ml) were sized as described previously [13] by dynamic light scattering measurements made at 23°C using light from a helium/neon laser (model 125; Spectra-Physics) that was focused into an isothermally jacketed scattering chamber. Deteriosome-free cytosol was also examined by light scattering to confirm that all of the microvesicles had been removed by the filtration procedure.

Permeability Measurements

The permeability of deteriosomes and microsomes was determined by measuring the retention of methylamine as described by Schumaker and Sze [24]. The isolated deteriosomes or microsomes (1 mg protein/ml) were pre-incubated in 50 mM $NaHCO_3$–300 mM sucrose, pH 6.0, for 2 h at 4°C. After pre-incubation, 100 μl of deteriosome (100 μg protein) or 100 μl of microsomes (100 μg protein) were added to 900 μl of 50 mM $NaHCO_3$–300 mM sucrose, pH 8.0, containing ^{14}C-methylamine (1,000,000 cpm) in the presence or absence of 0.1% Triton X-100. The mixture was vortexed and incubated for up to 20 min. At specified times during incubation, samples were filtered through an Amicon filter (0.05 μm in diameter). The filter was washed twice with 3 ml of 50 mM $NaHCO_3$–300 mM sucrose, pH 8.0, air-dried, and counted for radioactivity. The results are presented as cpm per mg protein.

Phospholipase A_2 Activity Assay

Phospholipase A_2 activity of deteriosomes was determined by measuring the release of free fatty acids from phosphatidylcholine [28]. Liposomes, which were made by adding 2.86 μg of radiolabelled phosphatidylcholine (PC; 1-stearoyl-2-[1-^{14}C] arachidonyl phosphatidylcholine; SA, 58 mCi/mmol; Amersham, Arlington Heights, IL) to 60 μg of egg L-α-phosphatidylcholine (Sigma), were incubated at 37°C for 30 min with microsomal membranes (100 μg protein), deteriosomes (100 μg protein), or deteriosome-free cytosol (100 μg protein) in assay buffer (50 mM Hepes, pH 7.0, 150 mM KCl, 0.2 mM EGTA, 1 mM $MgCl_2$, and 0.01% Triton X-100). In some cases, a calcium buffer was used in the assay (0.2 mM EGTA, 1 mM $MgCl_2$, and 250 μM $CaCl_2$), which generates a free Ca^{2+} concentration in the assay mixture of 40 μM [4,19]. After incubation, lipids were extracted and separated on TLC plate [28]. The radioactivity associated with the arachidonic acid band on the TLC plate was measured using a liquid scintillation counter.

RESULTS

Characterization of Deteriosomes

The post-microsomal supernatant (130,000*g* for 1 h) contained phospholipid and protein that were not sedimented during subsequent protracted high-speed centrifugation (250,000*g* for 12 h). When this resulting supernatant was concentrated and washed three times with 2 volumes of homogenizing buffer by filtration through a 1,000 kDa cut-off filter, the phospholipid and some of the proteins did not pass through the filter. This indicates that the lipids and proteins were assembled in lipid-protein microvesicles (deteriosomes). This was supported by the size distribution histogram of deteriosomes obtained by dynamic light scattering (Fig. 1). Deteriosomes ranged from 0.05 μm to 0.11 μm in radius with an average radius of 0.08 μm. However, the size distribution histogram of the corresponding filtrate (deteriosome-free cytosol) obtained by dynamic light scattering showed that there was no vesicle structure in the filtrate (data not shown). These results confirm that deteriosomes do not pass through the 1000 kDa cut-off filter. As shown previously for plant deteriosomes [31], electron microscopy of negatively stained liver deteriosomes indicated that they are spherical in nature (data not shown).

Thin-layer chromatography of lipid extracts from these deteriosomes indicated that they are enriched in neutral lipids by comparison with membranes (Fig. 2, lanes 1, 2). These neutral lipids include free fatty acids, long-chain aldehydes, and long-chain hydrocarbons, all of which are phospholipid catabolites [31]. Densitometer scans of the thin-layer chromatograms of lipid extracts from microsomal membranes and deteriosomes showed that the phospholipid degradation products (free fatty acids, long-chain aldehydes, and long-chain hydrocarbons) account for 25% of microsomal membrane lipid and 74% of deteriosome lipid isolated from the same tissue.

Protein composition analysis by SDS-PAGE indicated that the polypeptide profiles of microsomal membranes and deteriosomes are also different (Fig. 3, lanes 1, 2). In particular, the high molecular weight peptides (> 90 Kda), detectable in membrane gels, were not evident in gels of deteriosomes. There are also low molecular weight peptide bands at the bottom of the deteriosome gel that are not present in the membrane gel. These results indicate that deteriosomes contain proportionally more low molecular weight peptides than do membranes, and these small peptides are presumably products of proteolysis.

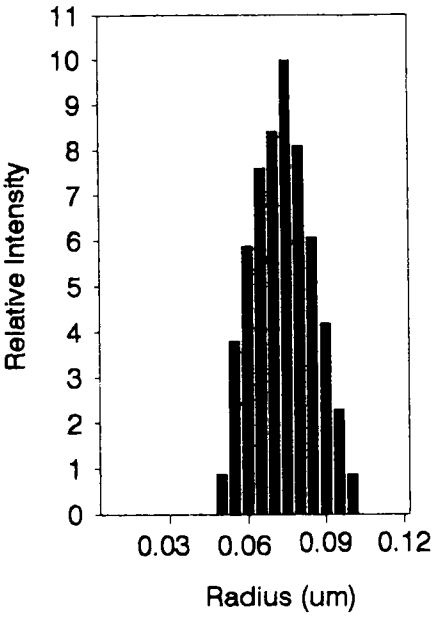

Fig. 1. Size distribution of deteriosomes obtained by dynamic light scattering.

Permeability of Deteriosomes

The unusual lipid composition of deteriosomes raised the possibility that they are highly perturbed and therefore could be more permeable than intact microsomes. To examine this possibility, permeability was determined by measuring retention of radiolabelled methylamine. When this probe is added outside the membrane or deteriosome vesicles (pH 8.0), methylamine is in its non-ionic molecular form and easily diffuses into the vesicles. Once inside where the pH is lower (pH 6.0), however, methylamine is protonated and can not diffuse out unless the vesicle is leaky. As shown in Figure 4, the methylamine retention in deteriosomes is only one-third as much as that in intact microsomes, suggesting that deteriosomes are more permeable than microsomes. However, when microsomes and deteriosomes were treated with 0.1% Triton X-100, a detergent that disturbs lipid bilayers, the retention of methylamine for both dropped to background level.

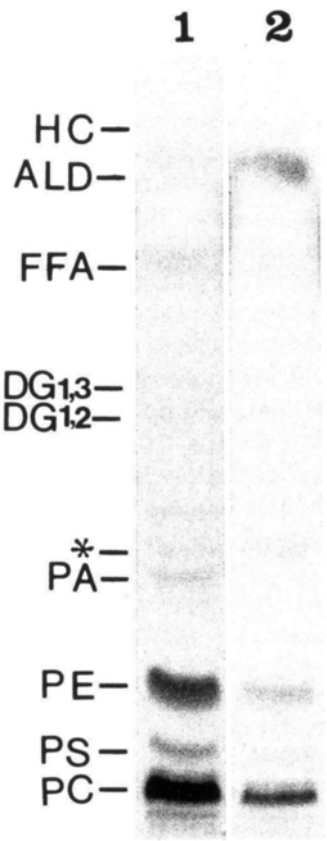

Fig. 2. Thin-layer chromatography of lipid extracts from microsomal membranes and deteriosomes. **Lane 1:** Microsomal membranes (0.5 mg protein equivalents). **Lane 2:** Deteriosomes (2 mg protein equivalents). HC, long-chain hydrocarbons; ALD, long-chain aldehydes; FFA, free fatty acids; $DG_{1,3}$, 1,3-diacylglycerol; $DG_{1,2}$, 1,2-diacylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Asterisk denotes unidentified lipid. The data are from one of three separate experiments, each showing the same results. The separated lipids were visualized with iodine vapour. (The same patterns were obtained when the separated lipids were visualized by sulphuric acid charring.) The densitometer scans of thin-layer chromatograms showed that neutral lipids account for 25% and 74% of the total lipids, respectively, for microsomal membranes and deteriosomes. Neutral lipids include diacylglycerols, free fatty acids, long-chain aldehydes, and long-chain hydrocarbons.

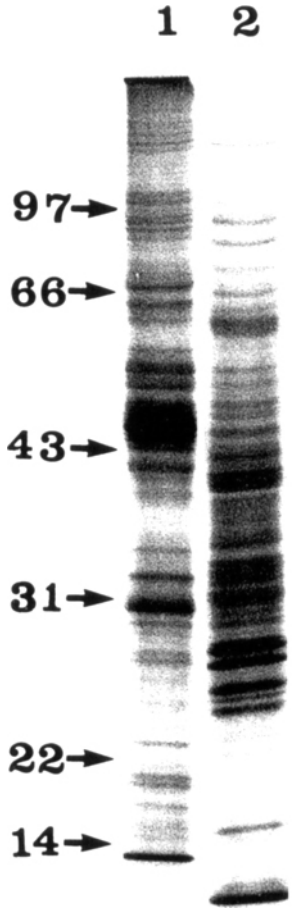

Fig. 3. SDS-PAGE of microsomal membranes and deteriosomes. Each lane contained 3 μg of protein. The gel was an 8–16% linear gradient and was stained with silver. **Lane 1:** Microsomal membranes. **Lane 2:** Deteriosomes. The data are from one of three separate experiments each showing the same results. Molecular weight markers (kDa) are indicated.

Phospholipase A_2 Activity

Phospholipase A_2 activity was expressed as the amount of free fatty acid released from radiolabelled phosphatidylcholine. As shown in Figure 5, phospholipase A_2 activity is present in microsomal membranes as well as in deteriosomes (Fig. 5A,B, respectively). Addition of Ca^{2+} augmented phospholipase A_2 activity. When Ca^{2+} was added to the assay buffer, enzyme activity was ~3.4-fold and ~3.3-fold greater than in microsomal membranes or deteriosomes with no Ca^{2+} added to the assay buffer. The specific activity of phospholipase A_2 is ~3.1-fold higher in deteriosomes than in the corresponding deteriosome-free cytosol (with 40 μM Ca^{2+}) (Fig. 5B,C). In terms of total cytosolic (post-microsomal) phospholipase A_2 activity, about 63% of the activity was associated with deteriosomes. Phospholipase A_2 activity in all of the fractions was completely inactivated by heat denaturation (data not shown).

DISCUSSION

The phenomenon of membrane microvesiculation under conditions of phospholipid catabolism was first reported for blood cells [1]. The formation of microvesicles and accompanying morphological changes in erythrocytes and lym-

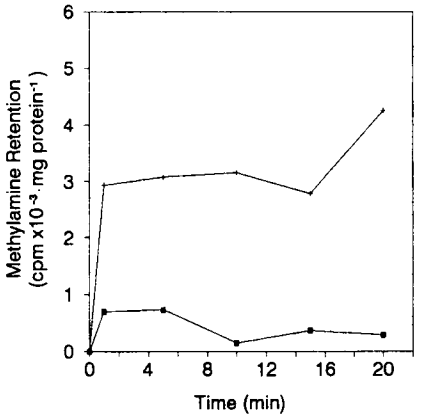

Fig. 4. Time course for the retention of methylamine by microsomes (+) and deteriosomes (■). Methylamine retention was taken to be the difference between the values obtained in the presence and absence of 0.1% Triton X-100. Data are from one of the three separate experiments showing the same results.

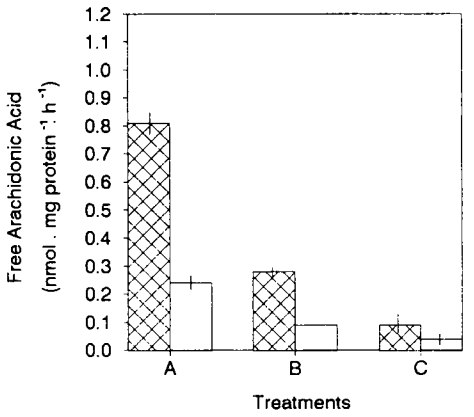

Fig. 5. Phospholipase A_2 activity measured as the release of radiolabelled free fatty acid from ^{14}C-phosphatidylcholine. The samples (100 μg protein equivalent) were incubated with radiolabelled phosphatidylcholine in liposomes for 30 min at 37°C in the presence (cross-hatched bars) or the absence of 40 μM Ca^{2+} (clear bars). **A**: Microsomal membranes. **B**: Deteriosomes. **C**: Deteriosome-free cytosol. Standard errors of the means are indicated; n = 3.

phocytes were observed when intact cells were treated in vitro with exogenous phospholipase C and Ca^{2+} in the presence of an ionophore [1,2]. Membrane microvesiculation was also observed in both plant and animal tissues under stress conditions, where the process of phospholipid catabolism was enhanced [11,20]. However, these microvesicles were either sedimentable or not isolated and characterized. In contrast, microvesicles (deteriosomes) from senescing bean cotyledons have been isolated and characterized and were found to be nonsedimentable [31]. The data in the present study show that nonsedimentable deteriosomes can also be isolated from animal tissue (i.e., rat liver).

Characterization of these deteriosomes indicates that they are chemically similar to the deteriosomes isolated from bean cotyledons in that they are enriched in protein and phospholipid catabolites [31]. The nonsedimentable lipids and proteins do not pass through a 1,000 kDa cut-off filter, which suggests that they are assembled in microvesicles. The nonsedimentable nature of deteriosomes presumably can be attributed to the fact that, in comparison with membranes, they are enriched in neutral lipids (phospholipid catabolites). Evidence for neutral lipid enrichment is found in the densitometry scans of the TLC plates. Neutral lipid accounted for 25% the total lipid extracted from the microsomal membranes, whereas the percentage of neutral lipid in the total lipid extracts of deteriosomes was 74%. The high percentage of neutral lipid also indicates that the deteriosome lipid bilayer is more perturbed than microsomal membranes, because these lipids have a propensity to promote non-bilayer configurations [25]. Examination of plant deteriosomes by wide angle X-ray diffraction revealed that they contain a mixture of liquid-crystalline and gel phase lipid domains [30]. The presence of gel phase lipid domains indicates that the microvesicles are leaky because of defects in the packing of bilayer molecules [30]. This concept is supported by the permeability results in the present study. It may also account in part for our inability to sediment the deteriosomes during the protracted centrifugation at 250,000*g* (12 h), because deteriosomes rapidly equilibrate with the buffer by reason of their high permeability. The probe used for these permeability studies, ^{14}C-methylamine, is present in undissociated form at pH 8.0 and can pass through the vesicles by diffusion. Preincubation of microsomes and deteriosomes in buffer at pH 6.0 acidifies the interior of these vesicles, in which the probe can be protonated once it diffuses in. The results obtained in the present study indicate that the probe remained at a higher level in microsomes than in deteriosomes suggesting that deteriosomes are leaky.

The fact that phospholipid catabolites are enriched in deteriosomes relative to membranes suggests that formation of deteriosomes by microvesiculation constitutes a pathway for removing destabilizing catabolites from membrane bi-

layers. The phenomenon of membrane microvesiculation can be induced in erythrocytes by treatment with Ca^{2+} or exogenous phospholipase C, which generates diacylglycerols [1,2]. Diacylglycerols are fusogenic and promote membrane microvesiculation. Although we did not find clear evidence for an accumulation of diacylglycerols in deteriosomes isolated from rat liver, this may be due to the fact that deteriosomes can be further catabolized [29]. The data in the present study are thus consistent with the view posed earlier [31] that deteriosomes represent an intermediate stage of membrane deterioration in that they serve as a vehicle for removing destabilizing membrane catabolites out of the bilayer for further processing.

To our knowledge, this is the first report of deteriosomes in animal tissue. Their presence in plant as well as animal tissues suggests that they may be ubiquitous. Moreover, the finding that deteriosomes possess phospholipase A_2 activity indicates that they may have organelle-like properties. Recent evidence indicates that this enzyme is Ca^{2+} sensitive and that it associates with membrane vesicles [6]. The present study also raises an important question concerning the assumption that cytosolic (post-microsomal) enzymes are all soluble. In rat liver, over 60% of the cytosolic phospholipase A_2 activity is actually associated with deteriosomes. These data suggest that at least some of the previously characterized cytosolic enzymes may not be truly soluble but rather associated with deteriosomes. A similar association with nonsedimentable deteriosomes may be found with other cytosolic enzymes.

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