

# Inhibition of 15-lipoxygenase leads to delayed organelle degradation in the reticulocyte

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**Abstract** Mammalian cells are characterized by an endomembrane system. Nevertheless, some cells lose these membranes during their terminal differentiation, e.g. red blood cells and lens fiber cells of the eye. 15-Lipoxygenase is believed to be critical for this membrane degradation. Here we use cultivated rabbit reticulocytes in the presence or absence of a lipoxygenase inhibitor to provide further evidence for the importance of 15-lipoxygenase for the *in vivo* degradation of mitochondria. We find that inhibitor treatment retarded mitochondrial degradation, as shown by persistence of marker proteins and by direct visualization of mitochondria by electron microscopy. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Lipoxygenase; Reticulocyte; Mitochondrion; Eicosatetraenoic acid; Organelle degradation; Red blood cell; Terminal differentiation

## 1. Introduction

One of the hallmarks of reticulocyte maturation is the degradation of the cell's organelles, the most abundant of which are mitochondria. Several degradation mechanisms have been proposed, most prominently autophagy [1] and cytosolic degradation [2]. 15-Lipoxygenase (15-LOX) has been proposed to take part in cytosolic organelle degradation in the reticulocyte [3]. The enzyme has recently been found to integrate specifically into organelle membranes, but not into the plasma membrane [4,5]. It thus permeabilizes organelles and creates access for the cytosolic proteolytic machinery, consisting mainly of the ubiquitin/proteasome system [6–8]. 15-LOX is only present at the reticulocyte stage of red blood cell maturation; until then 15-LOX mRNA is translationally inhibited by proteins binding to its 3'-UTR [9]. At the reticulocyte stage, however,

very large amounts of 15-LOX (up to 4 mg/ml of packed cells during experimental anemia) can be detected.

All of the aforementioned findings indicate that 15-LOX is in a perfect position to initiate the organelle degradation process. To establish whether the degradation of organelles during reticulocyte maturation is in fact lipoxygenase-dependent, we have cultured a reticulocyte-rich blood cell suspension in the presence or absence of the lipoxygenase inhibitor eicosatetraenoic acid (ETYA). ETYA is a dual-specificity inhibitor, affecting both lipoxygenases and cyclooxygenases. However, since no cyclooxygenases are present and the 15-LOX is the only lipoxygenase in the reticulocyte, this should not pose a problem in interpreting the results of our study. Previous studies utilizing lipoxygenase inhibitors have been restricted by detecting only amino acid release as a measure of overall proteolysis [10] or have only followed a single enzyme activity [11]. We have decided to use a more stringent approach including electron microscopy and Western blot analysis. We have found the inhibitor to diminish, but not to totally abrogate the organelle degradation process, supporting the concept that 15-LOX is a key enzyme in the cytosolic degradation of organelles during reticulocyte maturation. In addition to this cytosolic degradation, disposal of organelles via autophagic vacuoles occurs in parallel.

## 2. Materials and methods

Rabbits were made anemic by subcutaneous injection of phenylhydrazine (three doses of 12.5 mg/kg) according to Jackson and Hunt [12]. This protocol led to a decrease in cell number to about 25–40% of the non-anemic animal ( $0.8\text{--}2 \times 10^9/\text{ml}$  vs.  $4\text{--}6 \times 10^9/\text{ml}$ ). On day 8, rabbits were anesthetized by injection of ketamine/xylazine and intravenous injection of 2000 U of heparin prior to bleeding by heart puncture in accordance with the institutional animal protocol. To prevent clotting during incubation of the blood obtained, an additional 40 U of heparin was added per ml of blood. For incubation, the blood was diluted 1:20 in RPMI 1640 supplemented with 5% fetal bovine serum and incubated at 37°C in 6-well dishes (Nunc) for periods of up to 72 h. ETYA dissolved in dimethyl sulfoxide (DMSO) was added at a final concentration of 100 µM; this relatively high concentration of inhibitor was deemed necessary because of the enormous abundance of 15-LOX in the reticulocyte (overall cellular concentration around 50 µM). As a control an equal amount of DMSO (final concentration 0.3%) was added to parallel incubations. At the time points indicated, blood cells from individual wells were collected and processed as indicated below. Similar results to those presented here were obtained when other lipoxygenase inhibitors such as esculetin and salicyl hydroxamic acid were used (not shown).

Cell number and percentage of cells hemolyzed were determined for each sample. Only samples with less than 15% hemolysis, as judged by

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**Abbreviations:** 15-LOX, 15-lipoxygenase; ETYA, eicosatetraenoic acid; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; PDI, protein disulfide isomerase; MMCoAMutase, methylmalonyl coenzyme A mutase

absorption at 550 nm of a cell supernatant after centrifugation, were further analyzed. The percentage of reticulocytes in each sample was determined according to the manufacturer's protocol in a FACScan analyzer (Becton-Dickinson) after staining for RNA with thiazole orange. Typically, the samples contained between 35 and 60% reticulocytes at the time of bleeding. This value declined to about 10% after 72 h of incubation. To prepare samples for electron microscopy, 1 ml sample (corresponding to 50  $\mu$ l blood) was spun down in an Eppendorf centrifuge for 5 min at 5000 rpm. The pellet was resuspended in 1 ml freshly prepared 1% glutaraldehyde/0.1 M Na-cacodylate pH 7.5 and kept on ice for 20 min. After centrifuging for 5 min at 5000 rpm, the pellet was then washed with 0.1 M Na-cacodylate and resuspended in 30% sucrose/30% ethylene glycol/0.1 M Na-phosphate pH 7.5. Pellets were dehydrated with an ethanol series, infiltrated with increasing ratios of araldite 502 and Embed 812 resin (Electron Microscopy Sciences) to propylene oxide solvent, and the resin was left to harden at 60°C for 2 days. Ultrathin sections (0.7  $\mu$ m) were cut, floated onto grids and counterstained with lead citrate and uranyl acetate. Photomicrographs of all cells containing organelles or some internal structure were taken on a JEOL Temscan 100CX-II microscope.

To prepare samples for analysis by Western blotting, red blood cells were isolated by spinning the sample through 6 ml of Ficoll-Paque (Pharmacia) for 30 min at 1500 rpm. The red cell pellet was washed once with phosphate-buffered saline (PBS) and then hemolyzed by addition of 2.5 ml distilled H<sub>2</sub>O. The membrane fraction containing unbroken cells, plasma membranes and organelles was collected by centrifugation for 15 min at 100 000 rpm in a TLA 100.4 rotor (Beckman). The supernatant was precipitated at 66% ammonium sulfate to remove most of the hemoglobin, recombined with the pellet fraction, and analyzed by Western blotting for mitochondrial marker proteins. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), organelle marker proteins were detected by ECL detection (Amersham). To measure the membrane permeabilizing capacity of 15-LOX contained in the cytosolic fraction aliquots of the ammonium sulfate precipitated supernatants were incubated with ethylenediaminetetraacetate (EDTA)-high salt washed dog pancreas microsomes for 2 h at 37°C. A control incubation was carried out in the presence of buffer. The membranes were re-isolated by centrifugation through a 0.5-M sucrose cushion and aliquots of pellet and supernatant fractions were analyzed for content of the luminal endoplasmic reticulum (ER) protein disulfide isomerase (PDI) by SDS-PAGE and Western blotting.

### 3. Results

#### 3.1. The lipoxigenase fraction from reticulocytes cultured in the presence of ETYA no longer permeabilizes ER membranes

One prerequisite for studying the effect of lipoxigenase inhibitors on organelle degradation is to ascertain the effectiveness of the drug at inhibiting 15-LOX in the cellular setting. Taking advantage of the fact that ETYA is an irreversible inhibitor, we therefore isolated a cytosolic fraction from cells incubated in the presence or in the absence of inhibitor at various time points and assayed the activity of the lipoxigenase in vitro according to van Leyen et al. [4]. At each time point up to 72 h, the lipoxigenase from cells cultured in the absence of inhibitor was able to permeabilize ER membranes, as indicated by release of the luminal ER protein PDI into the supernatant. In contrast, utilizing the lipoxigenase from cells cultured in the presence of inhibitor greatly diminished this release (Fig. 1). This result demonstrates that ETYA effectively inhibits 15-LOX when added to cultured cells. On the other hand, as a negative control erythrocyte lysate is inactive in this assay (M. Wiedmann, unpublished).

#### 3.2. Morphology of reticulocytes cultured in vitro

In order to follow the reticulocyte maturation process, we

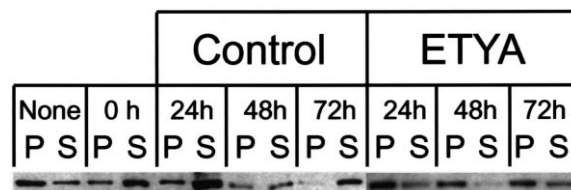


Fig. 1. 15-LOX isolated from reticulocytes cultured in the presence of ETYA can no longer permeabilize ER membranes. Lipoxigenase containing samples were prepared from the red cell fractions at the time points indicated according to Section 2 and incubated with EDTA-high salt washed dog pancreas microsomes. Control incubations were carried out in the presence of buffer (None) and with lipoxigenase containing fraction from freshly isolated blood cells (0h). After pelleting the membranes, aliquots of pellet (P) and supernatant (S) fractions were probed for PDI, a luminal ER protein, by SDS-PAGE and Western blotting. While the lipoxigenase contained in the supernatant fractions of cells incubated in the absence of lipoxigenase inhibitor is capable of permeabilizing ER membranes, the lipoxigenase from the inhibitor-treated cells leads to very little release of the marker protein, indicating inactivation of the lipoxigenase by ETYA.

analyzed the blood cells on the ultrastructural level. The percentage of reticulocytes at the time of bleeding, measured by RNA staining in a FACScan analyzer, typically ranged from 30 to 60% (data not shown). Moreover, the reticulocytes among these cells can be expected to be in various maturational stages, with early reticulocytes containing a multitude of organelles while later maturational stages are devoid of organelles. Previous studies have shown that these late reticulocytes cannot be readily differentiated from erythrocytes under the transmission electron microscope [13–15]. Consequently, when analyzed by electron microscopy, most cells exhibited a non-structured cytosol, indicating they are either erythrocytes or medium- to late-stage reticulocytes. Nucleated cells were only very rarely detected, reflecting the predominance of red cells over leukocytes. When organelles were found in non-nucleated cells (Fig. 2A–D), these were mostly mitochondria (M in Fig. 2A,B) and vacuoles (V in Fig. 2B–D), many of which contained organelle remnants. In addition, numerous ribosomes and polyribosomes were visible (small dark spots in Fig. 2C,D). Some siderosomes were also present (S in Fig. 2A,C).

After 48 h of culture in the absence of a lipoxigenase inhibitor, practically all cells exhibited the non-structured cytosol characteristic of late reticulocytes and erythrocytes, reflecting the in vitro maturation of these cells. In the rare cases where subcellular structures were visible (Fig. 2E–H), these were late-stage vacuoles either devoid of content (Fig. 2G,H) or containing severely damaged organellar remnants (Fig. 2E–H). No mitochondria or mitochondrial remnants could be detected in the cytosol. Also, no ribosomes or polyribosomal assemblies were visible, indicating the advanced maturational stage of these cells [16,17]. These findings correspond well to those of Steck and colleagues [14], who reported an effective maturation of rat reticulocytes cultured in vitro. In contrast, when cultured in the presence of ETYA, cells containing recognizable mitochondria could still readily be detected (Fig. 2I,J,K). Autophagic vacuoles containing organelles in various stages of degradation were also present (Fig. 2K,L). A number of these vacuoles contained mitochondria that appeared to be only mildly damaged. This could reflect an inhibition of the autophagic degradation pathway; alter-

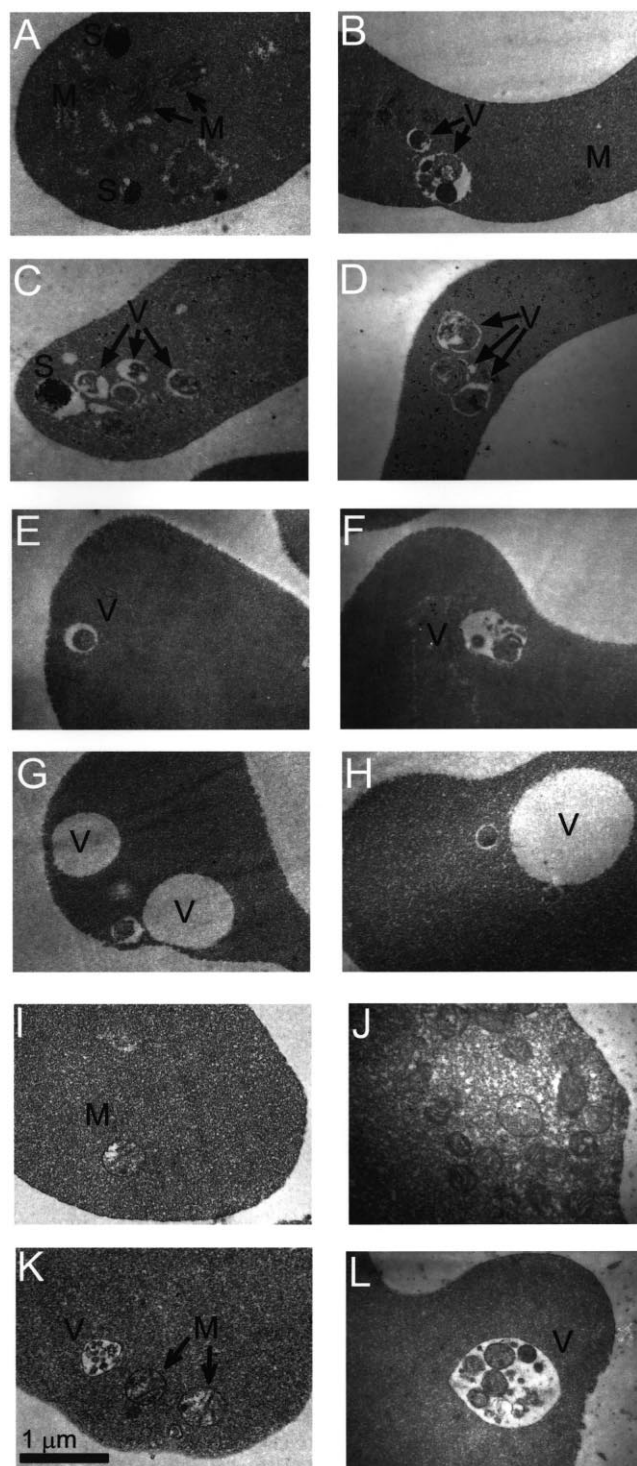


Fig. 2. Morphology of reticulocytes cultured in vitro. Blood cells were analyzed by electron microscopy to study the morphology of the reticulocytes obtained. Cells exhibiting some internal structure, indicating they are not erythrocytes, were selected under the electron microscope. A–D: Cells freshly isolated, prior to incubation. Besides mitochondria (M) and autophagic vacuoles (V), these cells also contained siderosomes (S). In C and D, ribosomes and polyribosomes are visible as electron-dense spots. E–H: Cells after 48 h incubation in the absence of lipoxygenase inhibitor. No mitochondria were detected, but vacuoles in some cases persisted. I–L: Cells after 48 h incubation in the presence of the lipoxygenase inhibitor ETYA. Cytosolic mitochondria as well as mitochondria enclosed in vacuoles are visible. Scale bar: 1  $\mu$ m.

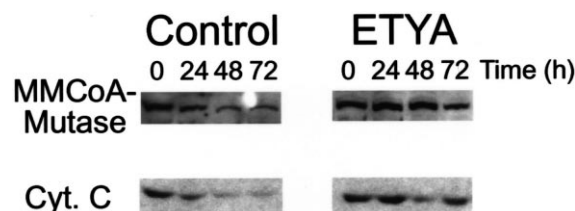


Fig. 3. Western blot analysis of red cells after incubation in the presence or absence of ETYA. After incubation for the times indicated, red cells were isolated by Ficoll separation to remove white blood cells and analyzed by Western blotting for mitochondrial marker proteins. Cytochrome *c* localizes to the intermembrane space, while MMCoAMutase is a matrix protein. Both proteins are degraded more rapidly in the cells incubated in the absence of ETYA, indicating a protective effect of ETYA towards mitochondria.

natively, these mitochondria may have only recently been taken up into a vacuole. At the same time, ribosomes and polyribosomes were absent. This demonstrates that the lipoxygenase inhibitor influenced only mitochondrial degradation without affecting other aspects of the maturation process.

### 3.3. Western blot analysis of red cells after incubation in the presence or absence of ETYA

The morphology of the cultured reticulocytes served as an indication that organelle degradation, and specifically that of mitochondria, is slowed down considerably when the cells are incubated in the presence of a lipoxygenase inhibitor. In order to determine the significance of these findings for individual proteins, we decided to analyze the cells for the presence of mitochondrial proteins by Western blotting. For this, the red blood cell fraction had to be isolated by centrifugation through a Ficoll cushion in order to eliminate leukocytes, which, despite their relatively low abundance in peripheral blood (ca. 0.1%) would falsify results owing to their large number of mitochondria. Samples at different time points were then probed with antibodies to mitochondrial marker proteins. Methylmalonyl coenzyme A mutase (MMCoAMutase) localizes to the mitochondrial matrix, while cytochrome *c* is localized in the intermembrane space. For both proteins, Western blotting of lysates from cells incubated in the absence of lipoxygenase inhibitor showed a rapid time-dependent decline of the signal, as could be expected from the electron micrographs. The residual signal apparent after 72 h could be due either to the proteins persisting after the organelle that once contained them has been degraded, or to contaminating residual leukocytes. In the cells incubated in the presence of the lipoxygenase inhibitor, the ECL signal for both proteins declined only slightly, indicating that the degradation of these proteins is delayed.

## 4. Discussion

We have analyzed the process of programmed organelle degradation in cultured reticulocytes. Analysis of control cultures by electron microscopy shows that after 48 h of incubation, no intact mitochondria can be detected on the ultrastructural level, and most cells exhibit the non-structured cytosol characteristic of late reticulocytes and mature erythrocytes. Occasionally, vacuoles with and without organellar remnants can be seen (Fig. 2E–H). These indicate that our cultured reticulocytes undergo a normal maturation process as charac-

terized in previous studies [14,18]. The process of reticulocyte maturation is also reflected in the time-dependent disappearance of the mitochondrial proteins, MMCoAMutase and cytochrome *c* (Fig. 3). In this case, low levels of these proteins persist even after 72 h, indicating the degradation process continues after the organelle itself has vanished. It is generally believed that reticulocyte maturation in the bloodstream occurs within 48 h [19]. Although this is supported by our ultrastructural findings, our Western blotting results indicate that full maturation, measured by protein turnover may not be completed within this time span. Conversely, this may reflect differences in the maturation process of reticulocytes in culture or in the live animal.

In the presence of ETYA, a lipoxygenase inhibitor, the organelle degradation process is delayed, consistent with the proposed function of the cytosolic 15-LOX in programmed organelle degradation. Since 15-LOX is no longer active under these conditions, the cytosolic pathway for disposal of organelles is disrupted, and relatively intact mitochondria can still be detected in the cytosol after 48 h of culture. In some cells, autophagic vacuoles containing mitochondria and mitochondrial remnants can also be observed. Since the lipoxygenase inhibitor would not be expected to inhibit this pathway, this presumably explains why some organelle degradation, and also some degradation of cytochrome *c* and MMCoAMutase, still occurs. It is likely that these mechanisms operate in parallel, providing for a backup system. The existence of two parallel pathways for organelle degradation may be the reason why mice in which the gene for the leukocyte-type 12-LOX (the presumed mouse orthologue of the 15-LOX-1 gene of rabbits and humans) has been disrupted do not exhibit gross abnormalities in their blood cells [20]. Our findings demonstrate that the cytosolic lipoxygenase pathway for organelle degradation is a major, but not the only means of organelle disposal in the reticulocyte.

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