A novel method for the study of autophagy: destruction of hepatocytic lysosomes, but not autophagosomes, by the photosensitizing porphyrin tetra(4-sulphonatophenyl)porphine

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A photoactivatable porphyrin, tetra(4-sulphonatophenyl)porphine (TPPS₄), was shown to accumulate in rat hepatocytes as a linear function of dose after intravenous injection, and to localize predominantly in hepatocytic lysosomes. A major fraction of the lysosomal enzymes acid phosphatase and *N*-acetyl- β -D-glucosaminidase was inactivated by TPPS₄ after 20 h of contact with the drug *in vivo* in the absence of photoactivation. On exposure of isolated hepatocytes to light, photoactivated TPPS₄ caused additional inactivation of the lysosomal enzymes as well as inactivation of intralysosomal lactate dehydrogenase (LDH), a cytosolic enzyme that accumulated in lysosomes as a result of autophagy during a 2 h incubation of hepatocytes at 37 °C in the

dark (in the presence of the proteinase inhibitor leupeptin to prevent degradation of intralysosomal LDH). Photoactivation of TPPS₄ also induced lysosomal rupture, with a loss of lysosomal enzymes, autophagocytosed LDH, endocytosed ¹²⁵I-tyramine-cellobiose-asialo-orosomucoid and TPPS₄ from the lysosomes. However, LDH-containing autophagosomes, accumulated in the presence of vinblastine (a microtubule inhibitor used to prevent the fusion of lysosomes with autophagosomes or endosomes), were not affected by TPPS₄. TPPS₄ may thus be useful as a selective lysosomal (or endosomal) perturbant in the study of autophagic–endocytic–lysosomal interactions.

INTRODUCTION

Autophagy is one of the main cellular mechanisms for the degradation of intracellular macromolecules and organelles [1]. In the first step of this process, a part of the cytoplasm is enclosed by a sequestering membrane, resulting in the formation of an early autophagic vacuole called an autophagosome. Most research so far has concentrated on the regulation of this initial sequestration step. Amino acids, hormones, cyclic nucleotides and protein kinases have been identified as important regulators [1]. However, little is known about the early autophagic vacuoles. It has been suggested that autophagosomes can fuse with endosomes to form a prelysosomal acidic vacuole, for which the name amphisome has been proposed [2]. Eventually the early autophagic vacuoles deliver their contents to lysosomes, probably by fusing with the latter. The sequestered material is thereby exposed to the acidic interior of the lysosomes and subjected to degradation by the hydrolytic enzymes. The fusion with lysosomes can be suppressed by vinblastine, a potent inhibitor of microtubular function [3].

We have investigated the possibility of using a photoactivatable porphyrin, tetra(4-sulphonatophenyl)porphine (TPPS₄), as a lysosomal perturbant to study autophagic–lysosomal vacuole interactions. After intravenous injection of TPPS₄, a substantial fraction of the drug has been shown to accumulate in the rodent liver [4], probably by endocytosis. Intracellularly, TPPS₄ localizes inside lysosomes, and on exposure to light it catalyses the formation of hyper-reactive singlet oxygen, which can cause both inactivation of lysosomal enzymes and rupture of the lysosomes [5]. The present results show that TPPS₄ taken up by rat liver

cells *in vivo* can be activated by subsequent illumination of isolated hepatocytes, resulting in the inactivation of lysosomal enzymes and of a cytosolic enzyme (lactate dehydrogenase, LDH) delivered to lysosomes by autophagy. Furthermore light-activated TPPS₄ induces lysosomal rupture, whereas LDH-containing autophagosomes, accumulated in the presence of vinblastine, are unaffected by TPPS₄. Selective inactivation and destruction of lysosomes by light-activated TPPS₄ thus seems to be a useful method for the study of the interrelation between autophagy, endocytosis and lysosomal degradation.

MATERIALS AND METHODS

Injection of TPPS₄ and ¹²⁵I-tyramine-cellobiose-asialo-orosomucoid (¹²⁵I-TC-AOM)

Male Wistar rats (250–350 g) were given a single intravenous injection of TPPS₄ (10 mg/ml in PBS) at doses of 0–250 mg/kg body mass, 20 h before hepatocyte isolation (intraperitoneal injections gave similar results). Within this dose range there was a linear relation between the amount of TPPS₄ injected and the amount of TPPS₄ subsequently recovered in isolated hepatocytes (e.g. 0.18 μ g of TPPS₄/mg cellular wet mass after injection of 250 mg of TPPS₄/kg body mass). No signs of toxicity were observed, even at the highest TPPS₄ dose tested. Administration of doses higher than 250 mg/kg was not attempted, because the amount of fluid needed to dissolve such large amounts of TPPS₄ would have made the injection volume intolerably large (at concentrations over 10 mg/ml, TPPS₄ tended to precipitate).

When ¹²⁵I-TC-AOM was used, 1.5 $\times$ 10⁷ c.p.m. (20 μ l of a 0.5 mg/ml solution containing 750 000 c.p.m./ μ l) was mixed

Abbreviations used: ACP, acid phosphatase; β -AGA, *N*-acetyl- β -D-glucosaminidase; GPN, glycyl-L-phenylalanine 2-naphthylamide; LDH, lactate dehydrogenase; ¹²⁵I-TC-AOM, ¹²⁵I-tyramine-cellobiose-asialo-orosomucoid; TPPS₄, tetra(4-sulphonatophenyl)porphine.

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with the TPPS₄ solution before injection. TPPS₄ did not affect the uptake of ¹²⁵I-TC-AOM *in vivo*. After the injection the animals were kept in the dark to prevent any toxicity or cell damage due to photoactivation of TPPS₄, and all further work with animals or cells was performed in a dark room.

Cell preparation and incubation

Hepatocytes were prepared from 18 h-starved rats by two-step collagenase perfusion [6]. The cells were incubated as suspensions (0.4 ml aliquots in shaking centrifuge tubes at 37 °C, 70–90 mg wet mass/ml) in suspension buffer [6] fortified with pyruvate (20 mM) and Mg²⁺ (2 mM), i.e. an amino acid-free medium that induces a maximal rate of autophagy [7,8]. Whole liver samples were taken by tying and cutting off a small piece from the distal end of a liver lobe after 30 s of perfusion with perfusion buffer [6]. The sample was immediately transferred to ice-cold homogenization buffer [0.25 M sucrose/1 mM EDTA/10 mM Hepes (pH 7.3)], cut into smaller fragments and homogenized as described below.

Cell disruption and separation of sedimentable cell components from cytosol

After incubation, the cells were washed twice in 10% (w/v) unbuffered, electrolyte-free sucrose. The samples were resuspended in 1 ml of 10% unbuffered sucrose and disrupted by a single high-voltage (2 kV/cm) pulse [9,10]. The disrupted material was layered on top of an ice-cold density cushion of phosphate-buffered metrizamide/sucrose [8% (w/v) metrizamide/0.05% sucrose/1 mM dithiothreitol/1 mM EDTA/50 mM potassium phosphate (pH 7.5)] and centrifuged at 7000 *g* and 4 °C for 30 min. The sedimenting cell corpses, containing all autophagolysosomal vacuoles, were thereby separated from the cytosol [11]. The metrizamide/sucrose cushion was aspirated and the pellet resuspended in 50 mM potassium phosphate buffer, pH 7.5, and frozen and thawed before enzyme/radioactivity measurements were made.

Cell fractionation

After incubation and washing, each sample was resuspended in 0.5 ml of 10% unbuffered sucrose, and four samples were pooled before electrodissruption. The disrupted material was mixed with an equal volume of buffered isotonic sucrose [0.25 M sucrose/1 mM EDTA/10 mM Hepes (pH 7.3)] and homogenized by 15 strokes (or 20 strokes for whole liver samples) in a Dounce homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 2000 *g* in an SS-34 rotor (Sorvall) for 2 min to obtain a nuclear pellet and a postnuclear supernatant. The nuclear pellet was washed once and the combined supernatants (post-nuclear fraction) were layered on top of an ice-cold density cushion of phosphate-buffered metrizamide/sucrose and centrifuged at 47000 *g* (20000 rev./min) and 4 °C for 60 min. The metrizamide/sucrose cushion was aspirated and the pellet (large-granule fraction) carefully resuspended in 2 ml of buffered isotonic sucrose.

Analytical sucrose density gradients were prepared by first layering 5.5 ml of 63% (w/v) buffered sucrose underneath 5.5 ml of 15% (w/v) buffered sucrose (both sucrose solutions containing 1 mM EDTA and 10 mM Hepes, pH 7.3), in Beckman Ultra-Clear tubes (14 ml nominal volume). The two solutions were then mixed in two steps in a Biocomp Gradient Master (Nycomed Pharma, Oslo, Norway). The standard parameters used were 5 min, 60 °C and 30 rev./min (step 1) and 25 s, 80 °C and 25 rev./min (step 2). The densities ranged from 1.04 g/ml in the top fraction

to 1.23 g/ml at the bottom of the gradient. An aliquot (1.4 ml) of the large-granule fraction was layered on top of the gradient, which was then centrifuged at 285000 *g* (40000 rev./min) in an SW40/9E 2800 rotor in a Beckman L8-70M Ultracentrifuge at 4 °C for 1 h 45 min. After centrifugation the gradient was divided into 20 fractions of 0.63 ml by upward displacement by using Maxidenz as displacement fluid. The densities of the fractions were calculated from the refractive indices.

Light exposure

The cells were exposed to light either before or after electrodissruption, or after the cell corpses had been separated from the cytosol by centrifugation through metrizamide/sucrose. In all cases the whole sample, resuspended in 1 ml of 10% sucrose in a 3 cm dish, was exposed to the light from a bank of four fluorescent tubes (model 3026; Applied Photophysics, London, U.K.). The fluence rate reaching the cells was 36 W/m² with a maximum at approx. 405 nm. Light exposure was done at 4 °C for a maximum of 16 min (standard time), with shaking of the dishes every 2 or 4 min to ensure that all cells were exposed to equal fluences of light.

Fluorescence microscopy

Freshly isolated hepatocytes (70000 cells) in 5 ml of Dulbecco's modified Eagle's medium/Ham's medium F12 (1:1), containing 45 µg of collagen, were seeded out in each 5 cm dish [12]. After 2 h of incubation in the dark, some of the cells were exposed to light for 16 min; all cells were then washed twice in PBS and a coverglass was gently put on top. The cells were examined in a Zeiss Axioplan microscope equipped with epifluorescence, with an HBO/100 W mercury lamp for excitation. Fluorescent images were recorded by means of a cooled charge-coupled device camera (TE2; Astromed, Cambridge, U.K.), connected to a computer for digital image processing and storage. The microscope was equipped with a 390–440 nm bandpass excitation filter, a 470 nm dichroic beam splitter and a 610 nm long-pass filter.

Measurement of TPPS₄ enzyme activities and radioactivity

For TPPS₄ measurements, 100 µl of cell suspension (approx. 10 mg of cells) was mixed with 900 µl of ice-cold 80% (v/v) methanol. After centrifugation at 7000 *g* for 10 min, the absorbance at 415 nm (maximum) of the supernatant was read in a Beckman model 26 spectrophotometer. The amount of TPPS₄ was calculated from a standard curve (where 1 µg/ml TPPS₄ gave an *A*₄₁₅ value of 0.394) and corrected for cellular background (0.020).

LDH [13] and acid phosphatase (ACP) [14] were determined spectrophotometrically in a Technicon RA-1000 autoanalyser, with kits for automated analysis from Boehringer Mannheim. *N*-Acetyl-β-D-glucosaminidase (β-AGA; hexosaminidase) was determined by the method of Barrett [15]. Radioactivities were measured in a γ-counter (1261 Multigamma; LKB Wallac, Oulu, Finland).

Reagents

TPPS₄ was provided by Porphyrin Products (Logan, UT, U.S.A.). Nycodenz, Maxidenz and metrizamide were purchased from Nycomed Pharma (Oslo, Norway); leupeptin was from Peptide Institute (Osaka, Japan). Protein assay kits were purchased from Bio-Rad (Munich, Germany). Kits for automated analysis of LDH and ACP were obtained from Boehringer,

Mannheim, Germany. Vinblastine, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, cytochrome *c* and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Intracellular localization of TPPS₄

TPPS₄ taken up by the liver *in vivo* could be directly revealed in subsequently isolated hepatocytes by fluorescence microscopy, because TPPS₄ is a highly fluorescent molecule. To obtain a good resolution of the spatial distribution of TPPS₄, the cells were allowed to attach and flatten out on a collagen substratum (Figure 1), but the same pattern could also be seen with cells in suspension. In the dark, intracellular TPPS₄ was concentrated in vacuoles localized close to the nucleus (Figure 1C). There seemed to be little or no TPPS₄ in the cytosol, and no staining of peripheral endocytic vacuoles was observed. After 16 min of light exposure, the cells still seemed to have a normal morphology as indicated by phase-contrast microscopy (Figure 1B), but the intracellular TPPS₄ had left the vacuoles and was distributed throughout the cytosol (Figure 1D). The nuclei were also brightly stained, indicating that TPPS₄ had penetrated the nuclear envelope, but there was no selective concentration of TPPS₄ in the

Figure 1 Effect of light exposure on intracellular localization of TPPS₄ in hepatocytes

Hepatocytes were isolated from a rat given TPPS₄ (200 mg/kg body mass) intravenously 20 h earlier. The cells were plated on collagen-coated plastic dishes and allowed to attach to the substratum for 2 h. (A, C) Non-exposed cells; (B, D) cells exposed to light for 16 min. (A, B) phase contrast; (C, D) fluorescence. Hepatocytes have a heterogeneous size distribution owing to differences in ploidy and nuclearity, and show a variable degree of spreading in culture, neither size nor spreading being affected by TPPS₄. Magnification $\times 330$.

Figure 2 Subcellular distribution of lysosomes and TPPS₄ in livers and hepatocytes

Large-granule fractions (sedimenting at 2.8×10^6 g $\cdot$ min) were prepared from whole livers (A, B) or from isolated hepatocytes (C–F) of rats that had been given ¹²⁵I-TC-AOM only (A) or TPPS₄ in addition (200 mg/kg body mass) (B–F) intravenously 20 h earlier. All procedures were performed in the dark. Subcellular fractions were prepared immediately from whole livers, whereas the hepatocytes were incubated for 2 h at 37 °C without (C, D) or with leupeptin (E, F) and then electrodisrupted. Some of the electrodisrupted cells were exposed to blue light for 16 min (D, F). The large-granule fractions were further fractionated on sucrose density gradients, and ACP activity (○), ¹²⁵I radioactivity (▲) and TPPS₄ content (●) were measured in each fraction. Each fraction value is expressed as a percentage of the total value in the gradient (A, B, C, E) or in a parallel gradient from non-illuminated cells (D, E). Each panel represents a single experiment, representative of at least two performed.

nuclei as has been observed, for example, in human cervix carcinoma cells [16].

Subcellular distribution of TPPS₄

The intracellular localization of TPPS₄ was further examined by studying the distribution of large-granule fractions from whole livers and isolated hepatocytes on sucrose density gradients. A lysosomal enzyme, ACP and ¹²⁵I-TC-AOM, injected 20 h previously and degraded intralysosomally to acid-soluble, membrane-impermeant fragments [17], were used as lysosomal markers. As shown in Figure 2A, whole livers from control animals contained two types of lysosome, forming a major peak at 1.19 g/ml and a minor peak/shoulder at 1.15 g/ml. In the livers of animals given TPPS₄ intravenously 20 h before being killed, the lysosomal distribution was shifted markedly in favour

Figure 3 Light-independent effect of TPPS₄ on the activity of cellular and autophagocytosed LDH and lysosomal enzymes

Hepatocytes were isolated from rats given different amounts of TPPS₄ intravenously 20 h earlier. The cells were incubated for 2 h with 0.3 mM leupeptin, then electrodisrupted. LDH was measured in cell corpses and in total disrupted materials; ACP and β -AGA in disrupted materials only. (A) Total cellular LDH; (B) autophagically sequestered LDH in cell corpses; (C) ACP; (D) β -AGA. All enzyme activities are expressed as percentages of the mean value in control animals (activity per mg cellular wet mass). All 35 and 100 mg/kg values are the means for two animals; values for LDH and ACP at 200 mg/kg TPPS₄ are the means for four animals; other values represent individual rats.

of the peak at 1.15 g/ml (Figure 2B), suggesting either an interference with lysosomal function and the equilibrium between lysosomal subtypes [18,19], or simply a decreased lysosomal density reflecting swelling or other changes due to intralysosomal TPPS₄ accumulation. In addition, a lighter lysosomal peak at 1.11 g/ml was evident, probably representing lysosomes structurally altered by the TPPS₄ treatment. The distribution of TPPS₄ followed the distribution of the lysosomal markers closely (Figure 2B), suggesting that it was largely localized to the lysosomes.

Large-granule fractions prepared from freshly isolated hepatocytes showed the same lysosome distributions as when prepared from whole livers (control or TPPS₄-treated; results not shown), and the distributions were practically unaffected after a 2 h incubation of the cells in the dark (Figure 2C). On exposure of electrodisrupted cells to light, most of the lysosomes became non-sedimentable (presumably by TPPS₄-induced rupture) and disappeared from the gradient, leaving only a single small peak at approx. 1.17 g/ml (Figure 2D). The fact that the intralysosomal radioactivity (in ¹²⁵I-TC-AOM) disappeared along with the lysosomal marker enzyme indicates lysosome destruction, not just lysosomal enzyme inactivation.

Leupeptin, a proteinase inhibitor used in most of these experiments to preserve the intralysosomal activity of autophagocytosed LDH [11], made the lysosomes uniformly heavy even in TPPS₄-treated cells, causing them to sediment as a single skewed peak at 1.19 g/ml (Figure 2E). However, leupeptin did not prevent the destruction of lysosomes on illumination of disrupted cell materials (Figure 2F), in contrast with its protective

Figure 4 Light-independent effect of TPPS₄ on autophagic sequestration of LDH

Hepatocytes were isolated from untreated control rats (A) or rats given TPPS₄ (200 mg/kg body mass) intravenously 20 h earlier (B). The cells were incubated without additions (○), with 0.3 mM leupeptin (●), or with 50 μ M vinblastine (▲). After incubation the cells were washed and electrodisrupted, and cell corpses were separated from cytosol by centrifugation through a metrizamide density cushion. The amount of LDH in frozen/thawed cell corpses was measured and expressed as a percentage of total LDH in the cell. Each point is the mean $\pm$ S.E.M. for three independent experiments. All procedures were performed in a dark room.

effect against lysosome rupture induced by a lysosomal enzyme substrate [20].

Dark effect of TPPS₄

The effect of non-illuminated TPPS₄ (dark effect) *in vivo* was assessed by examining cells isolated from rats given an intravenous injection of TPPS₄ 20 h earlier. Phase-contrast microscopy did not reveal any TPPS₄-induced changes in cellular morphology (Figure 1A), and there was no increase in the fraction of dead or structurally damaged (leaky) cells in the suspension as measured by Trypan Blue exclusion or cellular LDH activity (Figure 3A). The activity of the LDH that had accumulated in autophagic-lysosomal vacuoles after 2 h of incubation with leupeptin, i.e. mainly in lysosomes, was only 25% lower in TPPS₄-loaded cells than in control cells (Figure 3B). However, lysosomal enzyme activities were already strongly reduced by TPPS₄: the activity of ACP was decreased by 90% at the highest doses of TPPS₄ (Figure 3C), and the activity of β -AGA was reduced by 60–70% (Figure 3D).

Although all procedures were performed in the dark, the spectrophotometric enzyme assays required some illumination that might have activated TPPS₄. However, assaying ACP at 500 nm, where TPPS₄ does not absorb light, instead of at the usual 405 nm, did not alter the results. Furthermore the addition of TPPS₄ to control samples (at 200 μ g/ml, i.e. 40-fold more than in samples from rats pretreated with maximal TPPS₄ doses) did not decrease the ACP activity significantly, suggesting that inactivation of the lysosomal enzymes had taken place before the assay.

The dark effect of TPPS₄ on autophagically sequestered LDH was further studied during a 2 h incubation of hepatocytes from rats that had received 200 mg/kg TPPS₄ (Figure 4). The activity of autophagocytosed LDH accumulating in the presence of leupeptin (●) was 20–25% lower in cells from TPPS₄-treated animals (Figure 4B) than in control cells (Figure 4A; see also Figure 3B), but there was no decrease in the activity of LDH

Figure 5 Effect of photoactivated TPPS₄ on intralysosomal ACP and β -AGA at different stages of cell dissociation

Hepatocytes, isolated from rats given TPPS₄ (25 mg/kg body mass) intravenously 20 h earlier, were exposed to light for the length of the time indicated at 4 °C, as intact cells (○), after electrodisruption (●) or as cytosol-free cell corpses after metrizamide density cushion centrifugation (△). In all cases ACP (A) and β -AGA (B) were measured in cell corpses and expressed as percentages of the initial value in cell corpses not exposed to light. The data represent a single experiment.

accumulating in the presence of vinblastine (▲). LDH is believed to accumulate mainly in lysosomes in the presence of leupeptin (required to prevent intralysosomal LDH degradation), but mainly prelysosomally, in autophagosomes, in the presence of vinblastine, which blocks autophagosome-lysosome fusion [3,21]. The data therefore suggest a partial inhibition of intralysosomal LDH by intralysosomal TPPS₄ in the dark, but no effect of TPPS₄ on autophagosomal LDH or on the autophagic sequestration process itself.

Inactivation and loss of intralysosomal enzymes after light-activation of TPPS₄ in isolated cells

Because one purpose of the present study was to develop a method for the selective destruction of lysosomes, the effect of TPPS₄ activation by exposure to light at various stages of cell fractionation was investigated, with lysosomal enzyme activity as an indicator of lysosomal affliction. TPPS₄-loaded intact hepatocytes, electrodisrupted hepatocytes or hepatocytic cell corpses (electrodisrupted hepatocytes without cytosol) were illuminated with blue light for up to 30 min. Figure 5A shows that illumination of intact hepatocytes for 30 min decreased the activity of ACP (as subsequently measured in cell corpses) by 90%. Illumination of electrodisrupted cells was even more effective, whereas much less effect was obtained on illumination of the cytosol-free cell corpses. The activity of another lysosomal enzyme, β -AGA, was not decreased as much as the ACP activity, but again the best effect was seen when electrodisrupted cells were illuminated (Figure 5B).

The effect of light-activated TPPS₄ on intralysosomal LDH (taken up by autophagy) was studied in a separate experiment where hepatocytes had been incubated in the presence of leupeptin to prevent intralysosomal proteolysis [11]. As shown in Figure 6, illumination of electrodisrupted, TPPS₄-loaded cells eliminated most of the sedimentable (largely intralysosomal), autophagocytosed LDH as indicated by density gradient analysis. Only specifically autophagocytosed LDH activity, sensitive to treatment of the intact cells with the autophagy inhibitor 3-methyladenine [22], is shown in Figure 6; the 3-methyladenine-resistant LDH background [23] was not decreased by illumination (results not shown).

Figure 6 Effect of photoactivated TPPS₄ on intralysosomal, autophagocytosed LDH

Hepatocytes, isolated from a rat given TPPS₄ (250 mg/kg body mass) intravenously 20 h earlier, were incubated in the dark for 2 h at 37 °C with 0.3 mM leupeptin in the absence or presence of 3-methyladenine, washed twice in 10% (w/v) sucrose, and electrodisrupted. The disrupted materials were either exposed to blue light for 16 min (○) or kept in the dark (●). Large-granule fractions (containing, on average, 2% of the total LDH in the disrupted material) were prepared and fractionated on sucrose density gradients. LDH activity was measured in the fractions and expressed as a percentage of the total LDH in the disrupted material corresponding to each gradient. The values given (means for two different experiments) represent the differences between gradients from cells incubated without and with the autophagy inhibitor 3-methyladenine, to obtain a specific measure of autophagocytosed LDH.

The loss of intralysosomal enzyme activities could be due to direct photochemical inactivation of enzymes by light-activated TPPS₄, to loss of enzyme from photochemically disrupted lysosomes before cell corpse isolation, or to a combination of both. In cell lysates (frozen/thawed cell corpse supernatants) exposed to light at 4 °C in the presence of TPPS₄ (5 µg/ml in 50 mM potassium phosphate buffer, pH 7.5), ACP and LDH were rapidly inactivated (essentially complete inactivation within 20 min); β -AGA more slowly (only 50% inactivated at 20 min). Light-activated TPPS₄ can thus clearly cause direct photochemical inactivation of enzymes. The degree of photosensitivity apparently depends on enzyme structure, accounting at least partly for the difference between ACP and β -AGA in terms of enzyme loss from light-exposed TPPS₄-containing cells or disrupted cell materials (Figure 5).

Loss of lysosomal integrity after photochemical treatment

Because the decrease in lysosomal enzyme activity could be due to leakage from lysosomes as well as to enzyme inactivation, an unequivocal measure of lysosomal damage was sought. A radioiodinated tyramine-cellobiose-asialo-orosomucoid conjugate, ¹²⁵I-TC-AOM, was included in the TPPS₄ solution injected *in vivo* 20 h before hepatocyte isolation. ¹²⁵I-TC-AOM is taken up by receptor-mediated endocytosis and degraded in lysosomes, where radiolabelled degradation products accumulate and serve as a non-enzymic lysosomal marker [17]. A loss of ¹²⁵I radioactivity from cell corpses can therefore be taken to indicate lysosomal rupture.

As shown in Figure 7A, increasing doses of TPPS₄ caused an increasing loss of ¹²⁵I radioactivity from illuminated cell corpses, indicating progressive lysosomal rupture. At the highest TPPS₄ dose (250 mg/kg), more than 80% of the ¹²⁵I radioactivity was lost within 4 min of illumination, suggesting that most of the lysosomes had been destroyed.

Figure 7 Effect of TPPS₄ dose and light exposure time on the inactivation of autophagocytosed LDH and lysosomal enzymes and on lysosomal integrity

Hepatocytes were isolated 20 h after giving rats intravenous injections of ¹²⁵I-TC-AOM and TPPS₄ at 0 (◆), 10 (○), 30 (●), 50 (□), 100 (■), 200 (△) or 250 (▲) mg/kg body mass. The cells were incubated for 2 h with 0.3 mM leupeptin, washed and electrodisrupted. The disrupted cells were exposed to light at 4 °C for the lengths of time indicated, and cell corpses were then isolated, frozen and thawed. ¹²⁵I radioactivity (A), β-AGA (B), ACP (C) and LDH (D) were measured in the cell corpses and expressed as percentages of the initial value in cell corpses not exposed to light. The data are from four different experiments, each curve representing a single experiment (animal).

A pronounced effect on intralysosomal enzyme activities was already evident at 10 mg/kg TPPS₄, which caused a 40 % decrease in β-AGA activity (Figure 7B), a 70 % decrease in ACP activity (Figure 7C) and a 60 % decrease in the activity of autophagocytosed LDH (Figure 7D), corresponding roughly to the relative sensitivities of these enzymes towards direct photochemical inactivation. Because there was only little effect on lysosomal integrity at this TPPS₄ dose (a 15 % loss of ¹²⁵I-TC-

AOM-derived radioactivity, Figure 7A), the decreases in enzyme activity seem largely to reflect inactivation rather than leakage. At higher TPPS₄ doses, a loss of enzyme from ruptured lysosomes could also contribute, as indicated by the extensive loss of ¹²⁵I-TC-AOM-derived radioactivity, eventually resulting in an approx. 80 % decrease in all enzyme activities. A comparison between enzyme activity and enzyme sedimentability suggested that the photochemically sensitive enzyme ACP was completely inactivated before lysosome rupture, whereas the more resistant enzyme β-AGA retained some activity that could leak out of the ruptured lysosomes (results not shown).

Light-activated TPPS₄ inflicts relatively little cytosolic damage

Because TPPS₄ inactivated the cytosol-derived enzyme LDH in the lysosomes, the possibility had to be considered that TPPS₄ released from ruptured (light-exposed) lysosomes (see Figure 1) might be capable of inactivating enzymes in the cytosol. The results in Figure 8 show that light exposure had no effect on total LDH in intact or electrodisrupted hepatocytes that did not contain TPPS₄ (Figure 8A), but in cells prepared from rats treated with TPPS₄ (35 mg/kg) the total LDH activity was decreased by 15 % after 16 min of illumination (Figure 8B). Only approx. one-third of this loss could have been due to inactivation of intralysosomal LDH (see Figure 4B), indicating that approx. 10 % of the cytosolic LDH had become inactivated. At a higher dose of TPPS₄ (100 mg/kg), another 10 % of the LDH was inactivated in intact, but not in disrupted, cells (Figure 8C). There thus seemed to be relatively little (approx. 10 %) damage to extralysosomal cell components when TPPS₄-containing cells were exposed to light after electrodisruption, although intralysosomal enzymes were inactivated with high efficiency. On illumination of intact cells that contained high concentrations of TPPS₄, the cytosolic damage was more extensive, but overt hepatocellular death was not observed even under these conditions.

Lack of effect of photoactivated TPPS₄ on autophagosomes

To investigate the possibility of an effect of photoactivated TPPS₄ on autophagosomes, hepatocytes were incubated with vinblastine (50 μM) rather than with leupeptin. Vinblastine has been shown to inhibit autophagic-lysosomal fusion and to cause the accumulation of autophagocytosed LDH prelysosomally in

Figure 8 Effect of photoactivated TPPS₄ on cytosolic (total hepatocytic) LDH

Hepatocytes were isolated from rats given an intravenous injection of TPPS₄ at (A) 0, (B) 35 or (C) 100 mg/kg body mass 20 h earlier. The cells were incubated for 2 h with 0.3 mM leupeptin, washed and resuspended in 10 % (w/v) sucrose, and exposed to light for the length of time indicated, either as intact cells (○) or after electrodisruption (●). The activity of LDH in the frozen/thawed disrupted cells was measured and expressed as a percentage of the initial value in electrodisrupted cells not exposed to light. Each panel represents one animal.

Figure 9 Effect of photoactivated TPPS₄ on autophagosomal and lysosomal LDH, and on lysosomal integrity and enzyme activity in vinblastine-treated hepatocytes

Hepatocytes were isolated 20 h after giving rats intravenous injections of TPPS₄ (0–250 mg/kg body mass). The cells were incubated for 2 h with 0.3 mM leupeptin (○) or 50 μ M vinblastine (●), washed and electrodisrupted. The disrupted materials were exposed to light at 4 °C for 16 min; the cell corpses were then isolated, frozen and thawed. ACP (A), β -AGA (B) or autophagocytosed LDH (C) was measured in the cell corpses and expressed as a percentage of the value in animals not treated with TPPS₄. Each point represents an individual animal.

autophagosomes [3,20]. As shown in Figure 9, the dose-dependent inhibitory effects of photoactivated TPPS₄ on the lysosomal enzymes ACP (Figure 9A) and β -AGA (Figure 9B) were similar in vinblastine-treated and leupeptin-treated cells, but the effect on autophagocytosed LDH was strikingly different. Whereas approx. 80 % of the autophagocytosed LDH became inactivated in leupeptin-treated cells, there was little or no inactivation in vinblastine-treated cells (Figure 9C). These results strongly suggest that autophagocytosed LDH must be translocated from autophagosomes to lysosomes before the enzyme can become inactivated by lysosome-resident TPPS₄.

The lysosomal enzyme activities in Figure 9 have been expressed as percentages of the values in control cells from untreated animals. Inactivation owing to the dark effect of TPPS₄ *in vivo* is therefore also included, which accounts for the high degree of overall enzyme inactivation (more than 90 % for β -AGA, and more than 99 % for ACP). The extent of lysosome rupture in these experiments, however, was the same as before, i.e. up to 85 %, as indicated by the loss of ¹²⁵I-TC-AOM-derived radioactivity from cell corpses (results not shown).

DISCUSSION

The mechanisms by which photoactivatable porphyrins can interfere with cellular functions are of interest not only from an experimental but also from a clinical point of view: porphyrins and related substances are currently used in photochemical treatment of certain forms of cancer [24]. The therapeutic use of porphyrins is in large measure based on their preferential retention in tumour tissue, but some normal tissues such as liver, kidney and spleen have also been shown to retain substantial amounts of porphyrins [4,25].

In the present study we found that only 1 % of the intravenously injected TPPS₄ had been taken up by the hepatocytes after 20 h, although the liver constitutes 2–3 % of the rat body mass. *In vivo*, TPPS₄ is bound to serum proteins, especially albumin [26], which in hepatocytes would be taken up by fluid-phase endocytosis, a relatively slow process in these cells [27]. After injection of TPPS₄ at 200 mg/kg, the concentration of TPPS₄ in the intracellular fluid would be approx. 0.3 mg/ml if the TPPS₄ were evenly distributed, assuming an intracellular fluid volume of 50 % of the cellular wet mass [28]. The distribution of TPPS₄ fluorescence in rat hepatocytes was clearly punctate, suggesting a vacuolar, at least partly lysosomal localization, as

previously observed in cultured human cells [29,30]. This assumption was corroborated by an analysis of organelle distributions on density gradients, where TPPS₄ coincided with lysosomal markers under all treatment conditions.

The absence of TPPS₄ from the cytosol would indicate that it had not entered the cells by passage across the plasma membrane, but rather that it had been taken up by endocytosis and transported to the lysosomes along the endocytic pathway. Apparently the presence of the bulky phenyl groups in TPPS₄ makes it membrane-impermeant: the corresponding non-phenylated tetrasulpho compound is capable of direct plasma membrane passage (K. Berg, unpublished work). Studies of TPPS₄ uptake by isolated hepatocytes have confirmed that this porphyrin is taken up by a mixture of fluid-phase and adsorptive endocytosis (K. Berg and P. O. Seglen, unpublished work). Because lysosomes occupy only 1–4 % of the hepatocyte volume [3], the concentration of TPPS₄ inside lysosomes (at a dose *in vivo* of 200 mg/kg) would probably be in excess of 10 mg/ml. *In vitro*, such high TPPS₄ concentrations caused precipitation, indicating that the degree of lysosomal TPPS₄ loading achieved might be close to optimal. In poly-D-glutamate endocytosis it has been suggested that the poor solubility of this polymer at low pH might cause it to precipitate on reaching the lysosomes [31], but the solubility of TPPS₄ is higher at low pH than in neutral solution ([29]; K. Berg, unpublished work), suggesting that it probably remains dissolved inside the lysosomes.

Our studies revealed relatively strong light-independent effects of TPPS₄ on lysosomal enzyme activities *in vivo*, in particular on ACP, which was inactivated by approx. 90 %. Previous authors have reported that other porphyrins, localized to the cytosol, can inactivate cytosolic enzymes in the dark [32–34]. As with photochemical inactivation, ACP was more sensitive than β -AGA, whereas sequestered (largely lysosomal) LDH, somewhat surprisingly, was more resistant than the lysosomal enzymes. The chemical mechanism of enzyme inactivation by TPPS₄ in the dark would therefore seem to differ from that used in the light, i.e. singlet oxygen generation [24].

Despite the ability of TPPS₄ to cause extensive dark inactivation of lysosomal enzymes, it did not affect hepatocytic autophagy: TPPS₄-loaded and control cells showed similar rates of autophagosomal LDH accumulation in the presence of vinblastine (included to prevent the autophagosomes from fusing with lysosomes [3]). In the absence of vinblastine, the addition of the proteinase inhibitor leupeptin allowed autophagocytosed

LDH to accumulate in the lysosomes [11]. The measured rate of LDH accumulation in TPPS₄-containing cells in the dark was only slightly below the control rate, indicating that the intralysosomal TPPS₄ had relatively little effect on LDH activity in the absence of photoactivation. In the absence of leupeptin, no accumulation of autophagocytosed LDH was seen in either control or TPPS₄-containing cells, indicating that the fusion between lysosomes and prelysosomal autophagic vacuoles proceeded normally in the presence of TPPS₄, and that autophagocytosed LDH became degraded intralysosomally. The TPPS₄-loaded lysosomes thus seem to have retained both their ability to fuse and their capacity to degrade LDH, despite the decreased lysosomal enzyme activities. Experiments with human cells have shown that lysosomal proteinases (cathepsins) can be inactivated by photoactivated TPPS₄ [34], but in the dark, cathepsin inactivation apparently does not occur to such an extent as to cause any detectable functional impairment of hepatocytic lysosomes.

Previous work has shown that photoactivated TPPS₄ not only inactivates lysosomal enzymes, but also induces lysosomal rupture in cultured human cells [34,35], and the present results show that this is also true in isolated rat hepatocytes. After rupture of hepatocytic lysosomes *in situ*, TPPS₄ diffused rapidly into the cytoplasm and became somewhat concentrated in the nucleus, as in the human cells [30]. Photochemical inactivation of intralysosomal enzymes occurred at lower TPPS₄ doses and shorter light exposure times than did lysosomal rupture. This would probably be related to the localization of TPPS₄ in the lysosomal lumen [30], its access to the membrane being restricted by the polysaccharides coating the inner surface of the lysosomes. Furthermore both the short half-life and the short diffusion distance of singlet oxygen [5] would limit its spatial activity range. Lysosomal enzymes that leak into the cytosol after lysosome rupture are already mostly inactivated, as shown for hepatocytic ACP in the present experiments and for β -AGA and cathepsin (L+B) in human cells [34], the leakage of active β -AGA from hepatocytic lysosomes being somewhat exceptional. Lysosomal enzymes therefore seem to contribute little to the cellular damage inflicted by TPPS₄-induced lysosome rupture; such damage is apparently a direct effect of the released TPPS₄ [30,34].

Some TPPS₄-induced cytosolic damage in the form of LDH inactivation could be detected after illumination, but the LDH sequestered in autophagosomes was not affected, and the cellular plasma membrane remained intact. With a moderate photochemical insult it was therefore possible to induce lysosomal enzyme inactivation and extensive lysosome destruction with a minimum of damage to the cytosol, and without compromising the integrity of other organelles such as the autophagosomes. In cultured human cells, moderate photochemical insults were able to destroy lysosomes extensively, with only small long-term effects on cell survival [30], but we have not investigated the ability of TPPS₄-treated hepatocytes to survive beyond the brief period of light exposure.

The extensive inactivation of autophagocytosed LDH by previously endocytosed, stored lysosomal TPPS₄ suggests that newly formed autophagic vacuoles fuse with pre-existing secondary lysosomes rather than with primary lysosomes derived from the Golgi apparatus. This is in accordance with recent morphological studies, which concluded that hepatocytic lysosomes are stable organelles, uniformly capable of engaging both in autophagy and endocytosis [21]. To what extent the endosomes of TPPS₄-loaded cells fuse with autophagosomes to form amphisomes [2,21] is a question not addressed by the present experiments, but one would at least expect the endosomes to be TPPS₄-

free and functional by the time of the experiments (20 h after injection with TPPS₄).

Treatment with vinblastine blocks both endosomal and lysosomal fusion processes in hepatocytes while allowing autophagy to proceed [3,21], thus causing an accumulation of LDH-containing autophagosomes in the cells [20]. Such vinblastine-induced autophagosomes are a convenient starting material for attempts to purify this organelle [20,36]. A major problem in autophagosome purification is to remove the lysosomes; previously we have used selective lysosome rupture by a lysosomal substrate, GPN (glycyl-L-phenylalanine 2-naphthylamide), for this purpose [20]. The ability of photoactivated TPPS₄ to destroy lysosomes selectively without affecting autophagosomes offers an interesting alternative for preparative as well as for analytical purposes. Whereas the GPN method is dependent on lysosomal enzyme activity (specifically, cathepsin C), the TPPS₄ method carries no such restrictions and can therefore be used in combination with enzyme inhibitors such as leupeptin, widely used in autophagy studies [11,37,38]. The need to perform TPPS₄ experiments largely in the dark is no major impediment: some dim light can be allowed during sample handling because high-intensity light exposure for several minutes is actually required for a good TPPS₄ effect. Furthermore, whereas GPN destroys only lysosomes, our preliminary studies have shown that TPPS₄ can be endocytosed by hepatocytes *in vitro*, thereby allowing destruction of endosomes as well. By using TPPS₄ in combination with inhibitors acting at different steps in the autophagic and endocytic pathways [39,40] this compound would seem to offer numerous experimental possibilities for the study of autophagic-endocytic-lysosomal interactions.

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REFERENCES

- Seglen, P. O. and Bohley, P. (1992) *Experientia* **48**, 158–172
- Gordon, P. B. and Seglen, P. O. (1988) *Biochem. Biophys. Res. Commun.* **151**, 40–47
- Kovács, A. L., Reith, A. and Seglen, P. O. (1982) *Exp. Cell Res.* **137**, 191–201
- Peng, Q., Evensen, J. F., Rimington, C. and Moan, J. (1987) *Cancer Lett.* **36**, 1–10
- Moan, J. and Berg, K. (1991) *Photochem. Photobiol.* **53**, 549–553
- Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- Woodside, K. H. and Mortimore, G. E. (1972) *J. Biol. Chem.* **247**, 6474–6481
- Seglen, P. O. and Gordon, P. B. (1984) *J. Cell Biol.* **99**, 435–444
- Gordon, P. B. and Seglen, P. O. (1982) *Exp. Cell Res.* **142**, 1–14
- Gordon, P. B. and Seglen, P. O. (1986) *Biomed. Biochim. Acta* **45**, 1635–1645
- Kopitz, J., Kisen, G. Ø., Gordon, P. B., Bohley, P. and Seglen, P. O. (1990) *J. Cell Biol.* **111**, 941–953
- Mossin, L., Blankson, H., Huitfeldt, H. and Seglen, P. O. (1994) *Exp. Cell Res.* **214**, 551–560
- Bergmeyer, H. U. and Berndt, E. (1974) *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim
- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Barrett, A. L. (1972) in *Lysosomes: A Laboratory Handbook* (Dingle, J. T., ed.), pp. 46–135, North-Holland, Amsterdam
- Berg, K., Madslien, K., Bommer, J. C., Oftebro, R., Winkelman, J. W. and Moan, J. (1991) *Photochem. Photobiol.* **53**, 203–210
- Berg, T. O., Strømhaug, P. E., Løvdal, T., Seglen, P. O. and Berg, T. (1994) *Biochem. J.* **300**, 229–235
- Seglen, P. O. and Solheim, A. E. (1985) *Exp. Cell Res.* **157**, 550–555
- Berg, T., Kindberg, G. M., Ford, T. and Blomhoff, R. (1985) *Exp. Cell Res.* **161**, 285–296
- Berg, T. O., Strømhaug, P. E., Berg, T. and Seglen, P. O. (1994) *Eur. J. Biochem.* **221**, 595–602
- Fengsrud, M., Roos, N., Berg, T., Liou, W., Slot, J. W. and Seglen, P. O. (1995) *Exp. Cell Res.* **221**, 504–519
- Seglen, P. O. and Gordon, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1889–1892
- Gordon, P. B., Høyvik, H. and Seglen, P. O. (1992) *Biochem. J.* **283**, 361–369
- Moan, J. and Berg, K. (1992) *Photochem. Photobiol.* **55**, 931–948

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- 25 Winkelman, J. (1962) *Cancer Res.* **22**, 589–596
- 26 Kongshaug, M., Moan, J. and Brown, S. B. (1989) *Br. J. Cancer* **59**, 184–188
- 27 Gordon, P. B., Høyvik, H. and Seglen, P. O. (1987) *Biochem. J.* **243**, 655–660
- 28 Seglen, P. O. and Solheim, A. E. (1978) *Eur. J. Biochem.* **85**, 15–25
- 29 Wessels, J. M., Strauss, W., Seidlitz, H. K., Rück, A. and Schneckenburger, H. (1992) *J. Photochem. Photobiol. B* **12**, 275–284
- 30 Berg, K. and Moan, J. (1995) *Cancer Lett.* **88**, 227–236
- 31 Kishore, B. K., Fuming, L., Maldague, P., Tulkens, P. M. and Courtoy, P. J. (1996) *Lab. Invest.* **74**, 1025–1037
- 32 Baldacci, S., Cima, R., Sicuro, T., Monici, M. and Cozzani, I. (1985) *Med. Biol. Environ.* **13**, 13–15
- 33 Freitas, I. and Novarina, A. (1987) *Photochem. Photobiol.* **46**, 699–706
- 34 Berg, K. and Moan, J. (1994) *Int. J. Cancer* **59**, 814–822
- 35 Berg, K., Prydz, K. and Moan, J. (1993) *Biochim. Biophys. Acta* **1158**, 300–306
- 36 Marzella, L., Ahlberg, J. and Glaumann, H. (1982) *J. Cell Biol.* **93**, 144–154
- 37 Furuno, K., Ishikawa, T. and Kato, K. (1982) *J. Biochem.* **91**, 1943–1950
- 38 Kominami, E., Hashida, S., Khairallah, E. A. and Katunuma, N. (1983) *J. Biol. Chem.* **258**, 6093–6100
- 39 Høyvik, H., Gordon, P. B., Berg, T. O., Strømhaug, P. E. and Seglen, P. O. (1991) *J. Cell Biol.* **113**, 1305–1312
- 40 Hølen, I., Strømhaug, P. E., Gordon, P. B., Fengsrud, M., Berg, T. O. and Seglen, P. O. (1995) *J. Biol. Chem.* **270**, 12823–12831

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