Structural Variants of poly(alkylcyanoacrylate) Nanoparticles Differentially Affect LC3 and Autophagic Cargo Degradation

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Nanoparticle drug carriers trigger a variety of cellular stress responses, including ER stress and antioxidant responses, but may also affect the intracellular degradative pathway autophagy. This can impose profound effects on drug delivery, cellular treatment responses, and nanoparticle cytotoxicity. We recently demonstrated that even small variations in the alkyl side chains of poly(alkylcyanoacrylate) (PACA) drug carrier nanoparticles, namely butyl (PBCA), ethylbutyl (PEBCA), or octyl (POCA), differentially induce ER stress and redox imbalance in human cell lines. Here, we systematically investigate how these PACA variants affect autophagy. Interestingly, treatment with PEBCA or POCA particles led to intracellular accumulation of the autophagosome marker LC3-II, but via different mechanisms. PEBCA induced an integrated stress response- and ATF4-mediated increase in *LC3B* mRNA, whereas POCA blocked autophagic degradation of LC3-II and long-lived proteins in bulk. PBCA also increased *LC3B* mRNA via the integrated stress response and ATF4, but unlike PEBCA, it inhibited LC3 lipidation and autophagic cargo degradation. Our data demonstrate that even subtle variations in NP structure can have profoundly different impacts on autophagy, and that careful monitoring of autophagic flux and cargo degradation is critical for drawing accurate conclusions. Our findings have important implications for the choice of PACA monomer in different therapeutic settings.

KEYWORDS: Poly(alkylcyanoacrylate), LC3, Autophagy, Integrated Stress Response, ATF4.

INTRODUCTION

Nanoparticles (NPs), here defined as having a hydrodynamic diameter of less than 200 nm, have huge expectations in biomedicine, for instance as drug carriers. However, to fully exploit the potential of NPs, detailed knowledge on the interactions between nanoscale material and biological systems is imperative. Emerging research indicates that NPs may affect the lysosomal intracellular degradation pathway called ‘autophagy,’ which is greek for ‘self eating’ [1–3]. Autophagy is an evolutionary conserved process that has fundamental functions in cellular homeostasis, metabolism, and survival, and important

roles in physiology and pathophysiology [4, 5], as recognized by the 2016 Nobel prize in Physiology or Medicine being awarded to Yoshimori Ohsumi for his work on autophagy. Understanding the details of how NPs influence autophagy-related proteins and the autophagic process is thus of great interest. One way that NPs may affect autophagy is through the induction of cellular stress responses, like oxidative stress and antioxidant responses, or endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), which are frequently triggered upon cellular binding or uptake of NPs [6–8] and linked to alterations in autophagic activity [9–11]. Moreover, NPs may interfere in a rather direct manner with the final stage of the autophagic process. Since the vast majority of NPs are unable to cross the plasma membrane directly, and are instead internalized by endocytosis [12],

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Figure 1. PACA NPs with very similar physicochemical characteristics (A) differentially affect LC3B protein levels (B, C, E, F, G), LC3 puncta (D), and *LC3B* transcription (C, G) in MDA-MB-231 cells. The data represent mean values ± SEM from three independent experiments; * $p < 0.05$; ** $p < 0.01$.

they interact and/or accumulate at the luminal side of the endolysosomal system. This may lead to lysosomal dysfunction and therefore aberrations in autophagy [1–3]. On the other hand, lysosomal stress may induce a transcriptional response to increase autophagy and lysosomal biogenesis via transcription factor EB (TFEB) [13, 14]. Hence, determining the effect of NPs on the autophagic pathway requires careful study.

Autophagy can be subdivided into macroautophagy, chaperone-mediated autophagy and microautophagy [15]. During macroautophagy, which is the most studied form, and hereafter referred to simply as ‘autophagy,’ the cytoplasmic cargo is sequestered into double-membrane vesicles termed ‘autophagosomes,’ which fuse with lysosomes to generate ‘autolysosomes,’ where the sequestered content is degraded by acidic hydrolases and subsequently released back to the cytoplasm [16]. During autophagy, the ubiquitin-like protein LC3 is covalently conjugated to the autophagic membrane, and this lipid-conjugated form of LC3, termed ‘lipidated LC3’ or ‘LC3-II,’ functions to recruit autophagic cargo [17]. Upon arrival to the autolysosomes, the LC3-II that resides at the inner autophagosome membrane is degraded along with the cargo. LC3 is the most frequently used marker of autophagosomes, and its flux to the autolysosomes is often used as a proxy for autophagic cargo flux [18]. Although a useful marker, it should be noted that LC3 also has a number of important non-autophagic functions in the cell [19], and autophagic cargo flux is not always accompanied by LC3 flux [20]. Autophagy is sometimes, but not always, accompanied by a transcriptional response that increases the expression levels of LC3 and other autophagy-related proteins [21]. This can, as indicated above, occur via TFEB in response to lysosomal stress [13, 14], or via other transcription factors, such as ATF4 and Nrf2, in response to a variety of other types of cellular stress [22–25].

We recently demonstrated that even minor alterations in NP composition can differentially affect cellular stress responses in human cell lines [26]. Three highly similar poly(alkylcyanoacrylate) (PACA) particles with slight differences in their alkyl side chains; ethylbutyl (PEBCA), butyl (PBCA), and octyl (POCA), differentially affected UPR and antioxidant responses, as well as the mode of cell death (the chemical structures of the side chains of the three monomers are shown in Fig. 1(A)). More detailed analyses in MDA-MB-231 breast cancer cells revealed that the PACA-induced cytotoxic stress responses were regulated by the transcription factors ATF4 and Nrf2. Here, we investigate whether PACA treatment affects autophagy, and whether autophagy regulation is linked to the previously detected PACA-induced stress responses. Interestingly, treatment with the three PACA variants differentially affected LC3B expression and LC3 lipidation. Moreover, autophagic cargo degradation was reduced in PBCA- and POCA-treated cells, but unaltered by PEBCA. Importantly,

none of the PACA NPs induced lysosomal dysfunction. Collectively, our results indicate that PEBCA increases LC3 expression without altering autophagic flux, PBCA inhibits autophagy prior to autophagosome formation, and POCA interferes with autophagy at a later, pre-lysosomal step.

MATERIALS AND METHODS

Materials

Hoechst 33342, valine, concanamycin A (ConA), pepstatin A, reduced glutathione (GSH), paraformaldehyde, mowiol, and trichloroacetic acid (TCA) were all from Sigma–Aldrich, St Louis, MO, USA. Torin1 was purchased from R&D Systems, MN, USA, [^{14}C]valine was from Perkin Elmer, Waltham, MA, USA, and L-leucyl-L-leucine methyl ester (LLOMe) was from Santa Cruz Biotechnology, TX, USA.

Preparation and Characterization of PACA NPs

The three variants of PACA NPs were prepared by the mini-emulsion polymerization method exactly as described [26]. The particles were characterized by dynamic and electrophoretic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK).

Cell Culture

MDA-MB-231, hTERT RPE-1 (hereafter referred to as RPE-1), and HeLa were from ATCC, Manassas, VA, USA and were cultured in RPMI, DMEM/F12 and DMEM, respectively. The medium was supplemented with GlutaMAX[™], 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (all from Sigma–Aldrich, St Louis, MO, USA) at 37 °C and 5% CO_2 . The cells were starved for amino acids by incubation in Earle’s balanced salt solution (EBSS, Cat. no 24010043, Thermo Fisher Scientific, Waltham, MA, USA). The Cathepsin D Activity Assay Kit (Fluorescence) (Abcam, Cambridge, UK) was performed according to the manufacturer’s descriptions. As a positive control the cathepsin D inhibitor pepstatin A (10 μM) was added directly to the cell lysate in one of the experiments. Fluorescence was measured by a Synergy2 plate reader (BioTek, Winooski, VT, USA).

siRNA Transfection

MDA-MB-231 cells were reverse transfected with small interfering RNAs (siRNAs, final concentration 10 nM) using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s description. Experiments were performed 48 h later. For knockdown of TFEB and TFE3, the following Silencer Select siRNAs from Ambion (Thermo Fisher Scientific, Waltham, MA, USA) were used: Silencer[®] Select Negative Control #1 (4390843), siTFEB-1 (ID 15495, siTFEB-2 (ID 15496),

or siTFE3 (ID 14031). For double knockdown of TFEB and TFE3, 10 nM of each oligo was combined, and control cells were transfected with 20 nM of control siRNA. For knockdown of ATF4, Silencer Select siATF4-1 (ID 1702) or siATF4-2 (ID 1704) from Ambion was transfected side-by-side with 10 nM of Silencer® Select Negative Control #1 (4390843). Quadruple knockdown of the integrated stress response kinases was obtained by a mixture of 10 nM siPERK (ID 18103), 10 nM siPKR (ID 11185), and 10 nM siHRI (ID 25822) from Ambion, plus 10 nM ON-TARGET plus siGCN2 (J-005314-06-0010) from Dharmacon, Lafayette, CO, USA. Control cells were transfected with 40 nM of Silencer® Select Negative Control #1 (4390843) from Ambion.

Immunoblotting

Treated cells were washed in cold phosphate buffered saline (PBS, pH 7.4) and lysed on ice in lysisbuffer (0.1 M NaCl, 10 mM Na₂HPO₄, pH 7.4, 1 mM EDTA, 1% Triton X-100, protease- and phosphatase inhibitors (Roche, Basel, Switzerland)). The lysates were cleared by centrifugation (14 000 ×g, 5 min). Nuclear-cytoplasmic fractionation was performed according to [27]. Briefly, cells were washed with cold PBS before cytoplasmic membranes, but not nuclear membranes, were disrupted by a 2 min exposure to NP40 (0.1% NP40, 0.1 M NaCl, 10 mM Na₂HPO₄, pH 7.4, 1 mM EDTA, protease- and phosphatase inhibitors (Roche, Basel, Switzerland)). After a brief centrifugation (14 000 ×g, 10 sec), the supernatant was transferred to a new tube (cytoplasmic fraction). The pellet was washed once in the NP40 buffer, lysed in 1× Laemmli sample buffer, boiled and sonicated briefly to reduce viscosity (nuclear fraction). All lysates were separated on 4–20% SDS-PAGE gels and transferred to PVDF membranes (both from Bio-Rad Laboratories, Hercules, CA, USA). The proteins were detected after overnight incubation with primary antibodies. For quantification of signal intensity the Quantity One software was used (Bio-Rad Laboratories, Hercules, CA, USA). Antibodies against the following proteins were used for immunoblotting: LC3B (#2775), p-S6K (#9205), S6K (#9202), p-4E-BP1 (#2855), 4E-BP1 (#9644), TFEB (#4240), Erk1/2 (#9102), histone 3-HRP (#5192), and eIF2α (#5324)—all from Cell Signaling Technology, Danvers, MA, USA, and vinculin (#sc-59803, Santa Cruz Biotechnology, TX, USA), and actin (#CLT9001, Cedarlane, Burlington, Canada).

RNA Isolation and Real-Time PCR

Total RNA was extracted, cDNA was synthesized and real-time PCR was run and analyzed as previously described [26]. The following QuantiTect® Primer Assays (QIAGEN, Hilden, Germany) were used: Hs_MAP1LC3B (QT00055069), Hs_TFEB (QT00069951), Hs_TFE3 (QT00041076), Hs_ATF4 (QT00074466), and Hs_TBP (QT00000721).

Microscopy

For confocal microscopy, cells seeded on glass coverslips were fixed in 4% paraformaldehyde for 15 min. After permeabilization in 0.5% Triton X-100 (5 min), the cells were blocked in 5% FBS in PBS (30 min), stained with an anti-LC3 antibody (#PM036, 1:500 dilution, MBL International, Woburn, MA, USA) or an anti-ATF4 antibody (#11815, 1:200 dilution, Cell Signaling Technology, Danvers, MA, USA) in 5% FBS in PBS (1 h), washed 3 times, and stained by Alexa488-conjugated donkey anti-rabbit (Jackson ImmunoResearch Europe Ltd, Cambridge, UK) for 30 min. Nuclei were stained with Hoechst (5 µg/ml, 10 min) and the coverslips were mounted in Mowiol. Images were taken by a Zeiss LSM780 confocal microscope (Carl Zeiss Microscopy, Jena, Germany) controlled by the ZEN 2010 software and equipped with a Zeiss Plan-Apochromat 63×/1.40 objective, a Laser diode 405-30 CW (405 nm), and an Ar-Laser Multiline (458/488/514 nm).

For detection of galectin-3 puncta, HeLa cells stably expressing mCherry-galectin-3 grown in glass bottom dishes (MatTek Corporation, Ashland, MA, USA) were imaged live on a Deltavision microscope (Applied Precision, Bratislava, Slovakia) using a 60× Plan-Apochromat (1.42 NA) objective. A 2× concentrated solution of LLOMe or PACA NPs (as indicated in Fig. 6(A)) was injected into the dish at $t = 5$ min and time-lapse images (5 z-sections 2.5 µm apart) were acquired every 1 min for a total time period of 1 h of LLOMe treatment, and every 5 min for a total of 10 h of PACA-treatment. The images were subjected to deconvolution and z-projection by using the softWoRx software (Applied Precision, Bratislava, Slovakia).

Long-Lived Protein Degradation (LLPD) Assay

Autophagic degradation of long-lived proteins was monitored by a radioactivity-based pulse-chase assay as described in detail in Ref. [28]. First, cells were incubated for 24 h (pulsed) with 0.135 µCi/ml [¹⁴C]valine in RPMI complete cell culture medium to incorporate radioactivity into newly synthesized proteins. Then the cells were subjected to two periods of chase in fresh medium containing 10 mM non-radioactive L-valine. During the first chase period (3 h), proteins defined as short-lived are degraded and the medium discarded, whereas during the second chase period (4 h) proteins defined as long-lived are degraded, and the liberated radioactivity assessed. To determine the autolysosomal contribution of LLPD, the 4 h chase was performed in the absence or presence of the v-ATPase inhibitor concanamycin A (ConA, 100 nM), which blocks acidification of autolysosomes and thereby inhibits lysosomal degradation. To determine whether the PACA NPs affect LLPD, the PACAs (or Torin 1 as a positive control), were present during the last, 4 h chase (i.e., sampling) period (see Fig. 5(H) for an experimental

timeline). The chase was stopped by adding TCA to the well to precipitate cellular protein, and the radioactivity in both supernatant and pellet was quantified by β -counting with a Liquid Scintillation Analyzer (Packard BioScience, Meriden, CT, USA). Percent degradation was assessed as the acid-soluble radioactivity (supernatant) divided by the total radioactivity (supernatant + pellet). The fraction of autolysosomal long-lived protein degradation was determined by subtracting the values obtained in the presence of ConA from the values obtained in the absence of ConA.

Statistical Analysis

The statistical significance between mean values from three independent experiments was determined by two-tailed unpaired Student's *t*-test. Unless otherwise indicated, the asterisks denote the statistical significances compared to the untreated control *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

Treatment with PACA NPs Differentially Affect *LC3B* mRNA- and LC3-II Protein Levels

The highly similar PACA NPs were produced by in situ polymerization of alkylcyanoacrylate monomers exactly as previously described [26]. The chemical structures of the monomers ethylbutyl- (PEBCA), butyl- (PBCA), or octylcyanoacrylate (POCA) are shown in Figure 1(A). The three PACA NPs were of similar size (137–157 nm) and were slightly negatively charged (zeta-potential -3 mV) (Fig. 1(A)).

As a first assessment of the effect of PACA NPs on autophagy, we determined the levels of lipidated (membrane-bound) LC3 in MDA-MB-231 cells treated for 4 h with increasing concentrations of PACA particles. The maximal concentration of each particle type was carefully selected in order to avoid overt cytotoxicity, as it is well-known that autophagy may be affected by cell death pathways [29, 30]. In accordance with our previously described cytotoxicity studies [26], we included concentrations up to 100 $\mu\text{g/ml}$ for PEBCA, whereas concentrations up to 25 $\mu\text{g/ml}$ were included for the PBCA and POCA particles. Within these particle concentrations no or very limited cytotoxicity is observed within the relevant time frame (up to 4 h) in MDA-MB-231 cells [26].

Strikingly, treatment with the three PACA NPs for 4 h differentially altered LC3-II protein levels (Figs. 1(B, C)). Whereas PBCA NPs hardly altered the levels of LC3-II, PEBCA and POCA elevated LC3-II levels in a dose-dependent manner, resulting in up to 2-fold and 3.5-fold increases, respectively (Figs. 1(B, C)). Intracellular concentration of lipidated LC3 at autophagic membranes can be observed as bright puncta by fluorescence microscopy [18]. In line with the western blot data, the

ability of PACA NPs to produce fluorescently labeled LC3-positive puncta was in the order POCA > PEBCA >> PBCA (Fig. 1(D)). The lack of increase in lipidated LC3 after 4 h of PBCA treatment was not due to a different kinetic influence on LC3 levels with this PACA variant, as neither longer (Fig. 1(E); 8 h) nor shorter (Fig. 1(F); 0.25–4 h) time points of PBCA treatment could produce any increase in LC3-II levels.

Interestingly, treatment with PEBCA NPs increased the levels of *LC3B* mRNA in a concentration-dependent manner, which correlated very well with the increase in LC3-II protein levels (Fig. 1(C)). A similar correlation between LC3-II protein- and *LC3B* mRNA expression was observed after 8 h of incubation (Fig. 1(G)). This suggests that the observed increase in LC3-II protein levels in PEBCA-treated cells may be mediated by increased transcription rather than stimulation of LC3 lipidation *per se*. Also PBCA treatment induced a strong increase in *LC3B* mRNA levels, especially at 12.5 $\mu\text{g/ml}$ (Fig. 1(C)). However, in contrast to PEBCA NPs, the increase in *LC3B* mRNA levels was not accompanied by an accumulation of LC3-II. This might suggest that PBCA treatment inhibits LC3 lipidation, or alternatively that it increases autophagic flux and thereby the clearance of LC3-II. A third scenario was observed in POCA-treated cells. Here the accumulation of LC3-II occurred without a concomitant increase in *LC3B* mRNA levels (Fig. 1(C)). Thus, it seems that the three types of PACA NPs differentially affect both *LC3B* mRNA levels and LC3 lipidation (see Fig. 6(C) for an overview of the different responses to PACA NP treatment that we observed with various autophagy measurements in the current study).

PACA Treatment Does Not Lead to a Prominent Inhibition of mTORC1 Activity

Since treatment with the three PACA NP variants differentially affected LC3-II levels, we wanted to assess how particle treatment affects the main negative regulator of autophagy, the mTORC1 complex [31]. mTORC1 activity is commonly determined by assessing the phosphorylation status of its downstream targets S6K and 4E-BP1. As expected, treatment of MDA-MB-231 cells with two well-known inducers of autophagy, amino acid starvation or the mTOR inhibitor Torin1, totally abolished the phosphorylation of both S6K and 4E-BP1 (Fig. 2(A)). In contrast, PACA treatment led to only partial, variable, or minor responses, depending on the PACA variant (Fig. 2(B)). Treatment with PEBCA and POCA led to a partial, gradual reduction of S6K phosphorylation, reaching 60% and 35% of control levels within 4 h, respectively (Figs. 2(B, C)). Surprisingly, there was a biphasic response of S6K phosphorylation to PBCA treatment. Within the first 30 min, p-S6K was reduced to 50% of control levels, followed by a gradual recovery that even exceeded the control level at 2 h of treatment (Figs. 2(B–C)), whereafter the signal declined. The biphasic response to PBCA

Figure 2. Treatment with PACA NPs does not lead to prominent inhibition of mTORC1 activity in MDA-MB-231 cells. (A) Positive controls validate immunodetection of mTORC1 activity. Representative immunoblots (B, E) and quantifications (C, D) of mTORC1 activity in cells treated with 25 $\mu\text{g/ml}$ of PACA NPs; mean values $\pm$ SEM from three independent experiments; * $p < 0.05$; ** $p < 0.01$.

treatment was however not reflected by the phosphorylation pattern of p-4E-BP1 (Figs. 2(B, D)), indicating that the PBCA-induced changes in p-S6K may not be related to regulation of mTORC1 activity. POCA- and PBCA treatment reduced the phosphorylation of 4E-BP1 by 35% and <20%, respectively, whereas the slight reduction observed upon PEBCA treatment did not reach statistical significance (Figs. 2(B, D)). Elevating the concentration of PEBCA to 100 $\mu\text{g/ml}$ did not increase the inhibitory effect on p-S6K and p-4E-BP1. On the contrary, the inhibition was weaker (Fig. 2(E)). Taken together, treatment with PEBCA and POCA at concentrations that induce LC3-II accumulation is accompanied by a modest inhibition of mTORC1 activity, as assessed by S6K and 4E-BP1 phosphorylation. mTORC1 inhibition is therefore not likely the sole explanation for the LC3-II increase observed with PEBCA and POCA.

PEBCA-Induced *LC3B* Transcription is Independent of TFEB/TFE3

Next, we aimed to elucidate whether other stress responses (i.e., unrelated to mTORC1) could be involved in the increased LC3 expression observed upon PACA treatment. We considered one candidate to be TFEB, which is a key transcriptional regulator of autophagy-related genes, for instance in response to lysosomal stress [13, 14]. TFEB activation, as evidenced by nuclear translocation, has been shown in cells treated with both silver [32] and polystyrene NPs [33]. For these experiments we focused on PEBCA NPs, as they induced *LC3B* mRNA expression in a manner that correlated with LC3-II levels across a wide concentration range. Treatment with the positive control Torin1 for 1.5 h activated TFEB, as revealed by dephosphorylation and translocation of TFEB to the nuclear fraction (Fig. 3(A)). Note the disappearance of the upper band

Figure 3. PEBCA elevates *LC3B* mRNA levels independently of TFEB/TFE3. (A) Nuclear translocation of TFEB in MDA-MB-231 cells treated with Torin1 (250 nM) or PEBCA NPs for 1.5 h. (B) *TFEB* and *TFE3* mRNA levels determined 48 h after transfection with non-targeting control siRNA or siRNAs targeting TFEB or TFE3. (C) The level of *LC3B* mRNA in cells that were depleted for TFEB and/or TFE3 for 48 h, and then treated with PEBCA NPs (50 µg/ml) for 4 h; mean values from two independent experiments.

from the cytoplasmic fraction, representing phosphorylated TFEB, and the concomitant appearance of the lower band, the non-phosphorylated TFEB, in the nuclear fraction. In contrast, when cells were treated with PEBCA NPs for 1.5 h at concentrations that significantly induced *LC3B* mRNA expression (12.5–25 µg/ml, Fig. 1(C)), minor or no effects were detected with respect to TFEB translocation (Fig. 3(A)). Only at the highest concentration of PEBCA (100 µg/ml), there was a partial depletion of cytoplasmic TFEB and appearance of TFEB in the nuclear fraction (Fig. 3(A)). To assess whether the limited nuclear translocation of TFEB observed at *LC3B*-inducing PEBCA concentrations was important for *LC3B* transcription, both TFEB and the related TFE3 were depleted by siRNAs. Despite highly efficient knockdown (Fig. 3(B)), *LC3B*

mRNA levels were still increased by PEBCA in cells depleted for TFEB alone, TFE3 alone, or both (Fig. 3(C)). Thus, it is unlikely that TFEB/TFE3 is responsible for regulating *LC3B* transcription in PEBCA-treated cells.

Treatment with PEBCA and PBCA Increases *LC3B* mRNA Levels in an ATF4-Dependent Manner

Since the PEBCA-mediated increase in *LC3B* mRNA levels did not seem to be regulated downstream of TFEB, we turned to the transcription factor ATF4. ATF4 has been shown to activate transcription of autophagy-related genes, including *LC3B* under various conditions [22, 23, 25], and we recently demonstrated accumulation of ATF4 upon treatment with PACA NPs [26]. In line with this, treatment of MDA-MB-231 cells with PEBCA and PBCA at concentrations that strongly induce *LC3B* mRNA (50 and 12.5 µg/ml, respectively), led to nuclear accumulation of endogenous ATF4 (Fig. 4(A)), indicating that ATF4-mediated transcription may be increased. Strikingly, depletion of ATF4 by two individual oligos that reduced the level of ATF4 by 82.9% ± 7.2% and 89.3% ± 6.1%, respectively, abolished both the PEBCA- and PBCA-mediated increases in *LC3B* mRNA levels (Fig. 4(B)). It is known that ATF4 expression can be regulated by the upstream stress sensing eIF2α kinases PERK, GCN2, HRI or PKR, constituting the *integrated stress response* (ISR) [34]. By simultaneous depletion of the four ISR kinases, we recently demonstrated that PACA-induced ATF4 accumulation was mediated via the ISR [26]. In line, simultaneous depletion of the ISR kinases abolished both PEBCA- and PBCA-mediated elevation of *LC3B* mRNA levels (Fig. 4(C)). PBCA-induced ATF4 accumulation was previously found to be downstream of redox imbalance and its accumulation to be prevented by excess antioxidants [26]. In a similar manner, also the PBCA-induced *LC3B* mRNA increase was abolished by excess GSH (Fig. 4(D)). Together our data indicate that treatment with PEBCA and PBCA NPs induce *LC3B* transcription as a response to ISR and ATF4 activation.

Treatment with PBCA and POCA Inhibit LC3 Flux and Autophagic Degradation of Long-Lived Proteins

Although ATF4-mediated transcription may explain the increased LC3 expression and elevated LC3-II levels in PEBCA-treated cells, it does not explain the variable effects observed on LC3-II levels in cells treated with the two other PACAs (PBCA and POCA). We therefore next aimed to better understand the regulation of lipidated LC3 levels in PACA-treated cells, and its relationship to autophagic activity. Although treatment with PEBCA or POCA increased both LC3-II levels and LC3 puncta (Fig. 1), this does not necessarily indicate increased rate of

Figure 4. PEBCA- and PBCA-mediated elevation of *LC3B* mRNA levels requires ATF4 and the ISR. (A) Nuclear translocation of ATF4 in MDA-MB-231 cells treated with PEBCA (50 µg/ml) or PBCA (12.5 µg/ml) for 4 h. (B, C) *LC3B* mRNA levels determined 48 h after transfection with non-targeting control siRNA, or two different siRNAs targeting ATF4, or a mixture of siRNAs targeting each of the four ISR kinases GCN2, PERK, HRI and PKR (mean values $\pm$ SEM from three independent experiments; * $p < 0.05$; ** $p < 0.01$). (D) PBCA-mediated elevation of *LC3B* mRNA levels is counteracted by excess GSH (one experiment performed in duplicate $\pm$ deviation from mean).

LC3 conjugation (lipidation) to the autophagosomal membrane *per se*, since it may alternatively result from a block in autolysosomal LC3-II degradation [18]. The ambiguity can be resolved through monitoring the autophagic LC3 carrier flux, often termed ‘LC3 flux,’ by treating cells (with PACA NPs) in the absence or presence of a lysosomal inhibitor (e.g., the v-ATPase inhibitor concanamycin A, ConA). ConA blocks acidification of autolysosomes and thereby inhibits LC3-II degradation [18]. If PACA treatment stimulates autophagosome formation, and thus LC3 lipidation, the levels of LC3-II should be further increased in the presence of ConA. In contrast, if NP treatment by itself inhibits autophagosomal degradation, or if it has no effect on autophagic degradation, no further change in LC3-II levels will be observed in the presence of ConA. Treatment of MDA-MB-231 cells with ConA alone for 4 h led to significant accumulation of LC3-II, as expected from inhibited LC3-II degradation (Figs. 5(A, B)), and co-treatment with the bona fide autophagy inducer Torin1 (Tor1) further elevated LC3-II accumulation in the presence of ConA, as expected from stimulation of autophagosome formation (Figs. 5(A, B)). In contrast to Torin1,

treatment with PEBCA for 4 h, even at high concentrations, did not further increase the levels of LC3-II in the presence of ConA (Figs. 5(A, B)). Moreover, although treatment with 25 µg/ml POCA led to very strong LC3-II accumulation (which was actually comparable to that observed with ConA alone), there was no further accumulation in the presence of ConA (Fig. 5(C)), indicating that POCA treatment itself blocks LC3-II degradation. Surprisingly, co-treatment with 25 µg/ml PBCA reduced the levels of LC3-II compared to that observed with ConA alone (Fig. 5(D)), indicating that LC3 lipidation is inhibited. Comparable results were observed when visualizing LC3 puncta in PACA-treated cells (Fig. 5(E)). Whereas Torin1 clearly increased puncta formation and intensity in the presence of ConA, treatment with PEBCA and POCA had no effect, and treatment with PBCA decreased the number of LC3 puncta (Fig. 5(E)).

Although Torin1-induced accumulation of LC3-II in ConA-treated MDA-MB-231 cells was highly reproducible, it was relatively moderate, i.e., 1.3-fold after 4 h of co-treatment (Fig. 5(B)). We therefore repeated these experiments in human retinal pigment epithelial (RPE-1)

cells, which are highly responsive to autophagy induction with starvation or Torin1 (our unpublished data). In RPE-1 cells, Torin1 treatment potently induced LC3 flux (1.5-fold increase compared to treatment with ConA alone already after 1 h, Figs. 5(F, G)). Despite this higher potential of autophagy induction, none of the PACA NPs could further induce the LC3-II levels compared to that observed with ConA alone (Figs. 5(F, G)). In conclusion, PACA treatment did not stimulate LC3 flux in either MDA-MB-231 nor in RPE-1 cells. In line with the data from MDA-MB-231 cells, PBCA co-treatment significantly reduced LC3 lipidation compared to that observed with ConA alone (Figs. 5(F, G)).

In addition to LC3 flux measurements, it is strongly advisable to monitor autophagic cargo degradation [18]. Long-lived proteins are prototypical autophagic cargo, and their degradation is considered a reliable measure of autophagic activity [18]. A schematic drawing of the long-lived protein degradation (LLPD) assay is shown in Figure 5(H) and a detailed description is found in Ref. [28]. After cellular incorporation of radiolabeled valine for 24 h, a 3 h chase period is introduced to wash out short-lived proteins. The degradation of long-lived

proteins is assessed during the subsequent 4 h of treatment, which is performed in the absence or presence of ConA to enable calculation of treatment effects on the fraction of autophagic-lysosomal LLPD. In line with the LC3 flux data, Torin1 treatment significantly induced autophagic LLPD (Fig. 5(I)), whereas no increase was detected upon treatment with the NPs. Rather, the autophagic degradation was significantly inhibited by PBCA and POCA (Fig. 5(I)). Taken together, although treatment with PBCA and POCA NPs has very different effects on LC3-II levels (no change with PBCA and strong increase with POCA—see Fig. 1), both treatments inhibit LC3 flux and autophagic cargo degradation. Moreover, although PEBCA increases *LC3B* mRNA and LC3-II levels (Fig. 1(C)), it does not alter LC3 flux or autophagic cargo degradation.

Autophagy-Inhibitory Concentrations of PACA NPs do not Evoke Lysosomal Perturbations

The effect of PBCA and POCA to reduce autophagic cargo degradation may either be due to inhibition of a pre-lysosomal step in the pathway (e.g., autophagosome formation or the fusion of autophagosomes with lysosomes),

Figure 5. Continued.

Figure 5. Treatment with PBCA and POCA NPs inhibits autophagic flux and cargo degradation. (A–D) Autophagic LC3 flux in MDA-MB-231 cells treated with the indicated concentrations of PACA NPs or 50 nM Torin1 (Tor1; a positive control) for 4 h in the absence or presence of the v-ATPase inhibitor concanamycin A (Con A, 100 nM). (E) LC3 puncta in MDA-MB-231 cells treated with PACA NPs or Torin1 for 4 h in the presence of Con A. (F–G) Autophagic LC3 flux in RPE-1 cells treated with PACA NPs for 1 h in the absence or presence of Con A. (H) Experimental timeline of the long-lived protein degradation (LLPD) assay. (I) Autophagic activity measured as lysosomal degradation of long-lived proteins in MDA-MB-231 cells treated with PACA NPs or Torin1 in the absence or presence of Con A. All bars in this figure show mean values $\pm$ SEM from three independent experiments; * p < 0.05; ** p < 0.01; *** p < 0.001.

or a direct detrimental effect of the NPs on lysosomal (and thus autolysosomal) function. The nuclear translocation of TFEB observed at 100 μ g/ml of PEBCA NPs may indeed suggest that PACA treatment induces lysosomal dysfunction to some extent. To more directly address this, we employed HeLa cells stably expressing mCherry-galactin-3, a commonly used system for detection of lysosomal permeabilization [35–37]. Notably, we recently determined that HeLa cells internalize PACA NPs to the same extent as MDA-MB-231 cells [26]. As expected, when the cells were treated with a positive control for lysosomal damage, L-leucyl-L-leucine methyl ester (LLOMe), a lysosomotropic detergent-like substance [36], numerous mCherry-galactin-3 puncta rapidly appeared (Fig. 6(A)). In contrast, even at high concentrations and prolonged treatment, the three PACA NPs did not induce evident galactin-3 staining above the background level (Fig. 6(A)). The only exception was galactin-3 puncta appearing in POCA-treated cells several hours after initiation of membrane blebbing, likely indicating secondary necrosis downstream of apoptosis. This is in line with our recent data demonstrating POCA-induced apoptosis across a panel of cell lines [26]. Of note, a slightly lower concentration of

PEBCA NPs was tested in HeLa cells than in MDA-MB-231 cells, as HeLa cells are more sensitive to PEBCA than MDA-MB-231 (see Figure 1 in Ref. [26]). To assess lysosomal homeostasis also in PACA-treated MDA-MB-231 cells, we next determined the activity of the lysosomal enzyme cathepsin D. Whereas the cathepsin D inhibitor pepstatin A fully abolished the enzymatic activity, no significant changes in cathepsin D activity were observed after 4 h of treatment with high concentrations of PACA NPs (Fig. 6(B)). Together, this indicates that treatment with PACA NPs does not cause lysosomal dysfunction. Thus, we conclude that PBCA-mediated inhibition of autophagic LLPD (Fig. 5(I)) is likely caused by a block at an early phase of the autophagic pathway (since PBCA reduced LC3 lipidation in the presence of ConA—Fig. 5), whereas POCA on the other hand, likely blocks autophagy at a late, pre-lysosomal step, e.g. by inhibiting the fusion of autophagosomes with lysosomes (thus leading to strong accumulation of LC3-II by itself and no further increase when co-treated with ConA—Figs. 5(F, G)). A simplified model of the PACA NPs differential effects on autophagy is shown in Figure 7.

Figure 6. Autophagy-inhibitory concentrations of PACA NPs do not induce gross lysosomal perturbations. (A) HeLa cells stably expressing mCherry-Galectin-3 were either treated with LLOMe (250 μ M) for up to 1 h, or with 50 μ g/ml of PEBCA, PBCA or POCA NPs for up to 10 h. Appearance of mCherry-Galectin-3 puncta was assessed in live cells by wide-field microscopy. Scale bar; 10 μ m. (B) Cathepsin D activity in MDA-MB-231 cells treated with PACA NPs for 4 h (mean values $\pm$ SEM from three independent experiments). (C) Overview of the PACA NPs' differential effects on LC3B and autophagic cargo degradation.

DISCUSSION

In the current study, we have demonstrated that treatment with highly similar NPs (PEBCA, PBCA, and POCA) can have very different impacts on autophagy in human cells. PEBKA treatment elevated LC3-II protein levels and upregulated *LC3B* mRNA expression via ISR-ATF4 stress signaling, but did not alter LC3 flux or autophagic cargo degradation. Treatment with PBCA also enhanced *LC3B* mRNA levels via the ISR-ATF4 axis, but, surprisingly, it displayed an inhibitory effect on LC3 lipida-tion and autophagic cargo degradation, indicating that PBCA blocks autophagy at an early step of the pathway. Finally, treatment with POCA led to strong accumu-lation of LC3-II, which was associated with reduced autophagic

degradation of LC3 as well as of autophagic cargo, indi-cating that POCA blocks autophagy at a late stage in the pathway, likely at the autophagosome-lysosome fusion step. Notably, none of the three PACA NPs appeared to induce lysosomal dysfunction, as indicated by lack of galectin-3 positivity and unaltered cathepsin D activity. Our results therefore suggest that the PACA NPs differen-tially affect LC3 and the autophagic pathway via inducing different stress responses rather than via direct lysoso-mal effects. Our demonstration of these different cellular effects depending on subtle variations in PACA NP com-position may have important implications for the choice of NP variant in drug delivery. The main findings and con-clusions of the study are summarized in Figure 7.

Figure 7. A simplified model of the PACA NPs' differential effects on LC3B and the various steps of autophagy. Lys: Lysosomes.

Various NPs have been reported to induce autophagy based on intracellular accumulation of autophagic structures or increased levels of LC3-II or LC3-positive puncta [3, 38, 39]. Our findings in cells treated with PEBKA or POCA NPs clearly demonstrate that an observed NP-induced accumulation of LC3-II or LC3 puncta does not necessarily reflect activation of autophagy, i.e., increased autophagosome formation, since it instead can be caused by a block at a late stage of the autophagic pathway (POCA) or increased transcription of *LC3B* (PEBKA). Moreover, our data from PBCA-treated cells reveal that although a certain nanomaterial does not induce conspicuous alterations in LC3-II levels, autophagic flux and degradation may still be affected. This demonstrates the importance of careful assessment of autophagic flux and cargo degradation in NP-treated cells before firm conclusions on autophagy can be drawn.

It was surprising to find that treatment with PBCA NPs inhibited LC3 lipidation. To the best of our knowledge, this is the first nanomaterial shown to affect this step of the autophagy pathway. The reduced lipidation was accompanied by lower autophagic cargo degradation, reflecting inhibition of the macroautophagic pathway. It is not clear why PBCA inhibits LC3 lipidation, whereas PEBKA and POCA do not. However, as one possibility, we consider that the phenomenon may be related to the prominent induction of oxidative stress that is observed upon treatment of cells with PBCA [26]. Although one study has proposed that oxidative stress increases the levels of lipidated LC3 and autophagy via reduction of ATG4 de-lipidation activity [40], a recent study has demonstrated more directly that LC3 lipidation and autophagy is limited by oxidative stress-mediated inhibition of ATG3 and ATG7 (factors that are crucial for LC3 lipidation) [41]. Relating to this and our previous [26] and current findings, it will be interesting to elucidate whether PBCA treatment inhibits LC3 lipidation via oxidative regulation of the lipidation machinery.

Interestingly, both PEBKA and PBCA rapidly increased *LC3B* mRNA levels in a manner that was dependent on ATF4 downstream of the ISR. However, accumulation of ATF4 protein levels does not appear to be sufficient for the rapid induction of *LC3B* transcription, since POCA, which rapidly induces ATF4 accumulation via ER stress [26], failed to increase *LC3B* transcription. Since POCA does not induce the same degree of oxidative stress as PEBKA and PBCA [26], and since PBCA-mediated elevation of *LC3B* mRNA levels was inhibited by GSH, it is tempting to speculate that the early *LC3B* increase observed with PEBKA and PBCA requires input from other oxidative stress-induced transcription factors in addition to ATF4. Such factors could for instance be FoxO1, Nrf2, ATF3, and CHOP, which are all known to co-regulate gene transcription together with ATF4 [22, 42–44]. It will be interesting in future studies to explore these and other possibilities to how PACAs induce *LC3* transcription, as well as to explore the functional implications of the increased LC3 expression. We consider that PEBKA- and PBCA-mediated induction of LC3 expression may be part of a futile autophagy-initiating response that is counteracted by the autophagy-inhibitory effects of the particles (Fig. 7). Alternatively, since LC3 has been shown to possess several autophagy-independent functions [19], the PACA-mediated increase in LC3 expression may be unrelated to autophagy.

Why do these highly similar PACA variants impose such different effects on autophagy and LC3? It has been reported that physicochemical characteristics such as NP size, surface charge, or dispersion affect the autophagic response to NPs [45]. However, since these factors also severely affect NP uptake [45], the observed NP-mediated effect on autophagy is often correlated with the load of nanomaterials in the endolysosomal system, and it may be challenging to identify additional underlying mechanisms. The PACA NPs tested in this study were of similar size, they were all negatively charged, and they were internalized to the same degree across a panel of cell lines [26]. Thus, the particle-specific effects that we observed on autophagy and on LC3 modulation are unlikely to be caused by differences in physical characteristics or uptake efficiency, and are rather related to more subtle differences. Interestingly, and supporting the data obtained in this study, a previous study reported that subtle differences in NP structure of multiwalled carbon nanotubes exerted differential effects on LC3. Treatment of U87 cells with a panel of 81 multiwalled carbon nanotubes synthesized with similar surface ligands, induced a highly variable number of LC3 puncta [46]. Unfortunately, assessment of autophagic flux or cargo degradation across this panel was not performed, thus it remains unknown whether the LC3 accumulation was associated with autophagosome formation, inhibition of LC3 flux, or transcriptional upregulation during the 24 h incubation. Intriguingly, another

study found that increasing hydrophobicity of surface-modified gold NPs correlated with increasing accumulation of LC3 puncta [47]. Since the hydrophobicity of the alkyl side chains of the three PACA NPs in this study is slightly different and increasing in the order PBCA < PEBKA < POCA, the POCA-induced LC3-II accumulation may potentially be explained by the slightly more hydrophobic surface of POCA NPs. Although all PACA variants are PEGylated, we cannot exclude that the differences in hydrophobicity may affect NP-cell interaction, by for instance shaping the composition of the protein corona that build up around NPs when incubated in serum-containing cellular growth medium [48]. Indeed, the protein corona was proposed to play a role when different effects on accumulation of LC3 puncta and LC3-II was observed in HeLa cells treated with NPs of equal size, but of different core material, namely Fe_3O_4 and gold NPs of 20 nm [49]. Thus, although the NP core material is not in direct contact with the cell, the material may indirectly affect cellular stress responses, like autophagy, by affecting the composition of the protein corona. Of note, it remains unknown whether the accumulation of LC3 puncta observed in the above studies was due to autophagy induction or blockage, or associated with a non-autophagic stress response. Whether and how differences in hydrophobicity or the protein corona composition can affect autophagy and other stress responses will be interesting questions to follow up in future studies.

In conclusion, our data demonstrate that even subtle differences in NP structure may have strikingly different impacts on autophagy and on the autophagy-related gene product LC3. Since autophagy is a key process for cellular homeostasis and in human physiology, detailed knowledge on how nanomaterials affect autophagy is important both for predicting toxic effects of nanomaterials in cells and organs, and to harness the full potential of nanomaterials in biomedicine.

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