Published in final edited form as:

Nanomedicine. 2013 February ; 9(2): 212–221. doi:10.1016/j.nano.2012.05.017.

AUTOPHAGY IS INVOLVED IN NANOALUMINA-INDUCED CEREBROVASCULAR TOXICITY

Lei Chen, MD, PhD¹, Bei Zhang, BS², and Michal Toborek, MD, PhD²

Molecular Neuroscience and Vascular Biology Laboratory, Department of Neurosurgery and Graduate Center for Nutritional Sciences, University of Kentucky Medical Center, Lexington, KY

Abstract

The current study focused on blood-brain barrier (BBB) disruption and neurovascular damage induced by engineered nanomaterials. Exposure to nanoalumina, but not to nanocarbon, induced a dose-dependent mitochondrial potential collapse, increased autophagy of brain endothelial cells, and decreased expression of tight junction proteins, occludin and claudin-5. Inhibition of autophagy by pretreatment with Wortmannin attenuated the effects of nanoalumina on decreased claudin-5 expression; however, it did not affect the disruption of occludin. These findings were confirmed in mice by administration of nanoalumina into the cerebral circulation. Systemic treatment with nanoalumina elevated autophagy-related genes and autophagic activity in the brain, decreased tight junction protein expression, and elevated BBB permeability. Finally, exposure to nanoalumina, but not to nanocarbon, increased brain infarct volume in mice subjected to a focal ischemic stroke model. Overall, our study reveals that autophagy constitutes an important mechanism involved in nanoalumina-induced neurovascular toxicity in the central nervous system.

Keywords

nanoalumina; autophagy; blood-brain barrier; central nervous system

INTRODUCTION

Recent years have seen rapid development of nanotechnology.¹ Its products, engineered nanomaterials (ENMs), have been engaged in an expanding spectrum of industry applications, consumer products, and medical fields.^{1,2} However, ENMs may execute adverse impacts on the environment and human health due to their unique small sizes, electrical charge, and surface reactivity.³⁻⁶ ENMs may readily penetrate the cell membrane, conjugate with cellular organelles, enter the nuclei, and disrupt normal cell functions.⁷ These events may trigger injurious responses at the cellular, subcellular and protein levels³.

© 2012 Elsevier Inc. All rights reserved.

Correspondence: Michal Toborek, MD, PhD, Molecular Neuroscience and Vascular Biology Laboratory, University of Miami School of Medicine, Gautier Bldg., Room 517, 1011 NW 15th Street, Miami, FL 33136, mtoborek@med.miami.edu, Phone number: 305-243-6261.

¹Current address: Department of Neuroscience, Mount Sinai School of Medicine, New York, NY

²Current address: Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami, Miami, FL

The authors do not declare any commercial current or past associations that might pose a potential, perceived, or real conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

With its wide industrial use, nanoalumina is one of the most abundantly manufactured ENMs. Aluminum acts as a disrupter of cell membranes and has been implicated as an etiological factor in several neurodegenerative diseases.^{4,5} There is evidence that exposure to aluminum may contribute to an increase in oxidative stress, inflammatory events, and the breakdown of the blood-brain barrier (BBB). Nevertheless, nanoalumina may have a different toxicity spectrum as compared to the bulk material. Indeed, our previous study indicated that nanoalumina displays much higher toxicity than alumina at micron-sizes and above.⁶

The normal function of the BBB is critical for the homeostasis of the central nervous system (CNS).⁷ The BBB is responsible for the active exchange of nutrients and metabolites between blood and the brain, as well as for the restrictive entry of immune cells and plasma contents into the brain. Growing evidence indicates that tight junctions are critically important in the regulation of the BBB during pathological insults to the CNS and multiple neurodegenerative diseases.

Autophagy is a highly conserved pathway of protein degradation involved in various cellular functions and cell death. Under normal conditions or low levels of cellular stress, it implements recycling of the cytoplasm and disposition of defective organelles. More persisting stressors, such as nutrient deprivation or toxic exposure, may convert these mechanisms into apoptosis or autophagic death.⁸ Due to the essential role of autophagy in the regulation of cellular stress, we examined its activity upon nanoalumina treatment. We demonstrate that cellular or systemic nanoalumina treatment induces mitochondrial dysfunction and autophagic activity, disrupts the BBB function, and exacerbates ischemic stroke.

METHODS

Cell culture and treatment factors

Well-characterized human cerebral microvascular endothelial cells (HCMEC/D3 cells) were cultured as described earlier.⁶ Nanoalumina (8-12 nm particle size, Alfa Aesar, Ward Hill, MA) stock solution was prepared in distilled water which was then diluted in culture medium (for cell culture studies) or PBS (for animal injections), and sonicated for 15 min before administration to avoid generation of aggregates. The particle size distribution was measured by dynamic light scattering (DLS) using DelsaTMNano C Particle Analyzer (Beckman Coulter, Indianapolis, IN). Nanoalumina suspensions showed a biphasic pattern including large agglomerates (~4,400 nm) and smaller fragments (<1,000 nm). Fifteen minute sonication resulted in a conversion of these aggregates into smaller components of average size 241 ± 27.6 nm.

To detect the cellular and tissue uptake, nanoalumina was labeled with Alizarin Red S (ARS, Sigma, St. Louis, MO) by adding nanoalumina stock solution to 0.9 mM ARS in 10 mM sodium phosphate solution (pH 7.4) and stirring for 15 min.⁹⁻¹⁰ ARS-labeled nanoalumina was detected under fluorescent microscope using Texas red channel (ex: 595 nm, em: 615 nm). ARS preserves ortho-substituted enediol ligand and hydroxyl groups of alizarin which enable its high affinity binding to nanoalumina. The labeling is stable as we detected a decrease in fluorescence of less than 10% after 7 days of maintaining ARS-labeled nanoalumina in an aqueous solution. Importantly, the labeling did not change the particle size distribution as compared to non-labeled nanoalumina.

In selected experiments, Wortmannin (a PI3-kinase inhibitor; 100 nM; Sigma) was used to inhibit autophagy or chloroquine (50 μ M; Sigma) was employed as the inducer of autophagosome accumulation.¹¹⁻¹²

Animal treatment, surgical procedures, and tissue preparation

All experiments were performed following the protocol approved by the Institutional Animal Care and Use Committee in strict accordance with the National Institutes of Health guidelines. C57BL/6 mice (males, 10-12 week old, Harlan, Indianapolis, IN) were subjected to carotid artery surgery to deliver vehicle or nanoalumina (1.25 mg/kg) or control substances directly into the cerebral circulation.¹³

The brains were removed, the cortexes were homogenized in distilled water, followed by determination of the ATP content by the ATP assay kit (Invitrogen, Carlsbad, CA). For immunostaining analyses, the brains were perfused with 4% formaldehyde in addition to saline, followed by sectioning into 20 μ m slices.¹³ The integrity of the BBB was assessed as described earlier,¹³ using sodium fluorescein as the marker. An experimental stroke model was induced by a permanent occlusion of the middle cerebral artery. Twenty-four hours later, the infarct volume was determined with 2,3,5-triphenyltetrazolium chloride (TTC) staining and calculated using ImageJ software.

Mitochondrial potential, lysosome formation, autophagic activity, and protein degradation assays

HCMEC were cultured on 2-well slides (BD, Bedford, MA), treated with nanoalumina or control factors, and stained with MitoTracker Red CMXRos Red (Invitrogen, Carlsbad, CA), and/or LysoTracker® Green (Invitrogen).^{11,14} The cultures were co-stained with monodansylcadaverine (MDC, Sigma) for 15 min²⁴ to detect autophagic vacuoles and observed under fluorescent microscope.

Protein degradation was examined using DQ-BSA (Invitrogen) as a substrate according to the manufacturer's instructions.

Expression of tight junction proteins and autophagy-related proteins

Expression of tight junction proteins (claudin-5 and occludin) and autophagy-related proteins (LC3 and p62) was evaluated by immunoblotting. Anti-claudin-5 and anti-occludin antibodies were from Invitrogen. Antibodies against LC3 and p62 were purchased from nanoTools (Teningen, Germany) and American Research Products (Belmont, MA), respectively.

Expression of LC3 was also analyzed by immunostaining on brain slices. The sections were co-stained for Factor VIII (marker of endothelial cells) or GFAP (marker of astrocytes). Both antibodies were purchased from Invitrogen and used at 1:400 dilutions.

Expression of autophagy-related genes

Autophagy-related genes were assessed in the brains by the mouse Autophagy PCR Array (Qiagen, Valencia, CA). The results were validated by determination of expression of LC3 mRNA by quantitative real-time PCR using commercially available primers and probes. LC3 mRNA levels were normalized to GAPDH mRNA expression.

Statistical Analysis

One-way ANOVA followed by Newman-Keuls Multiple Comparison Test was used to compare mean responses among the treatments. Statistical probability of $p < 0.05$ was considered significant.

RESULTS

Nanoalumina enters HCMEC and crosses the BBB to accumulate in the brain

ARS-nanoalumina (1 $\mu\text{g/mL}$, stained red) was detected in HCMEC following a 2 h treatment (Figure 1A, middle panel, arrows). After a 12 h exposure, ARS-nanoalumina was visible as aggregates in proximity to clustered mitochondria as assessed by staining with MitoTracker Green (Figure 1A, right panel, arrows).

In order to determine the fate of nanoalumina in the brain, ARS-nanoalumina was injected at the dose of 1.25 mg/kg into the cerebral circulation via the carotid artery. The brain endothelium and astrocytes were detected by staining for Factor VIII and GFAP, respectively. ARS-nanoalumina co-localized with the brain endothelium 1 h post-injection (Figure 1B, left panel, arrows) and was also present in astrocytes surrounding the cerebral vessels (Figure 1B, right panel, arrows). Twenty-four hours post-injection, ARS-nanoalumina exhibited a diffuse pattern in brain parenchyma in proximity to astrocytes (Figure 1C, left panel). The distribution of ARS-nanoalumina 1 week post-injection (Figure 1C, right panel) resulted in similar distribution patterns as that 24 h post-injection, indicating that nanoalumina accumulates in the brain and is not eliminated from the brain components.

Nanoalumina-induced loss of mitochondrial potential is associated with an increase in autophagic activity

Localization of nanoalumina to mitochondria seen in Figure 1A prompted us to determine its influence on mitochondrial potential. HCMEC were treated with nanoalumina (1 $\mu\text{g/mL}$) for 6 h and stained with MitoTracker Red. In addition, cells were stained with monodansylcadaverine (MDC) as a marker for autophagic vacuoles. Control cells revealed normal staining of mitochondria (Figure 2A, red staining) with minimal presence of autophagic vacuoles (Figure 2A, blue staining, arrows). Treatment with increasing doses of nanoalumina resulted in a progressive increase in diffused staining of mitochondria, indicating the loss of membrane potential. These changes were correlated with a notable increase in intensity of MDC staining. Treatment with Wortmannin, a specific PI3K inhibitor and inhibitor of autophagy, protected against the formation of autophagic vacuoles induced by 1 $\mu\text{g/mL}$ nanoalumina. Importantly, treatment with nanocarbon (nC, 1.2 $\mu\text{g/mL}$) did not alter mitochondrial potential or MDC-positive staining. The association of altered mitochondria and formation of autophagic vacuoles upon treatment with 1 $\mu\text{g/mL}$ nanoalumina for 6 h is shown in more detail in Figure 2B. Additional staining was performed with LysoTracker Green to determine the formation of autophagolysosomes. Merged images indicate co-localization of diffused mitochondria, lysosomes, and autophagic vesicles.

Mitochondria are the main producers of cellular ATP. Therefore, we determined ATP levels as a proof-of-principle that exposure to nanoalumina affects mitochondrial functions. As compared to vehicle-injected animals, nanoalumina treatment resulted in a dramatic decrease in ATP levels.

Nanoalumina stimulates expression of autophagic genes and proteins

Twenty-four hours after administration of nanoalumina (1.25 mg/kg) or vehicle via the carotid artery, mouse brains were subjected to mouse autophagy PCR array. Of the 84 autophagy-related genes examined, 13 genes demonstrated more than two fold increase. These genes, plus the gene encoding for microtubule-associated protein 1 light chain 3 (MAP1LC3 alpha and beta), are listed in Figure 3A. The elevated genes were interrelated when analyzed by the Gene Annotation Tool to Help Explain Relationships (GATHER). They were richly clustered in three Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathways, namely, path:mmu04210: Apoptosis, path:mmu04060: Cytokine-cytokine receptor interaction, and path:mmu04010: MAPK signaling pathway. Among the studied genes, only the tumor necrosis factor (ligand) superfamily member 10 was downregulated more than two-fold. To confirm the results, we determined expression of the LC3 gene by conventional real time PCR. As indicated in Figure 3B, LC3 mRNA was elevated in the brains of nanoalumina-exposed mice by 1.6-fold.

We also determined two frequently used autophagy marker proteins, namely, LC3 and p62. During autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine (LC3-II). LC3 binds to the LC3-interacting region (LIR) of p62 through the interaction of basic residues at its N-terminus with the acidic cluster on p62¹⁵. Complexes of p62 and LC3 localize to the autophagosomes and are degraded through the autophagy-lysosome system. As shown in Figure 4A, treatment of HCMEC with nanoalumina for 6 h induced an increase in both LC3 and p62 protein levels. Importantly, exposure to nanoalumina increased formation of LC3-II, which is consistent with the progression of autophagic processes. Treatment with nanocarbon also upregulated the levels of p62 and, although to a lesser extent than nanoalumina, increased the levels of LC3.

Similar to the results obtained in HCMEC, an increase in LC3 and p62 proteins was observed 24 h post-nanoalumina injection and remained elevated for as long as 30 days after nanoalumina administration (Figure 4B). It should be noted that anti-LC3 antibody used in this study can reliably detect LC3-I and LC3-II in cell culture samples but not in the brain tissues. Therefore, only one LC-3 band is visualized in Figure 4B.

Brain distribution of LC3-positive immunoreactivity is shown in Figure 4C. LC3 staining was prominent along the cerebral microvessels (identified by Factor VIII positive staining) at day 3 post-injection, with nanocarbon lacking such an effect. Thirty days post-nanoalumina administration, LC3 immunoreactivity was apparent throughout the brain parenchyma and was visible in proximity to astrocytes.

Nanoalumina-induced protein degradation is correlated with autophagolysosome formation

The effects of nanoalumina on protein degradation was assessed in HCMEC using DQ-BSA, a probe based on bovine serum albumin (BSA) labeled with red-fluorescent BODIPY TR-X dye. The vehicle-treated cells demonstrated minor basal levels of protein degradation (indicated as red fluorescence), while treatment with nanoalumina dose-dependently elevated activity of DQ-fluorescence (Figure 5A). Importantly, protein degradation activity was correlated with the formation of acidic vesicles, consistent with autophagolysosomes, as assessed by staining with LysoTracker Green. Treatment with nanocarbon at 1.2 $\mu\text{g/mL}$ also induced protein degradation activity and autophagolysosome-like structures, however, to a lesser degree as compared to equimolar concentration of nanoalumina.

While DQ-BSA serves as an exogenous probe for the overall protein degradation activity, we also assessed the effects of nanoalumina on the levels of occludin and claudin-5, tight junction proteins which regulate integrity and barrier function of the brain endothelium. Following a 6 h exposure to nanoalumina, a dose-dependent decrease in expression of occludin and claudin-5 expression was detected in HCMEC (Figure 5B). Pretreatment with Wortmannin (100 nM for 1 h), a PI-3 kinase inhibitor and blocker of autophagy, partially protected against nanoalumina-induced changes claudin-5 but not occludin expression.

Importantly, nanoalumina also decreased tight junction protein expression *in vivo*. A single dose of nanoalumina (1.25 mg/kg) administered via the carotid artery resulted in a gradual

decrease in occludin for up to 30 days post-injection when it reached the levels 50% below control values. The decrease in claudin-5 occurred earlier as compared to occludin (1 day vs. 3 days) and lasted for 5 days. While treatment with nanocarbon also decreased claudin-5 and occludin levels, these effects were less advanced as compared to nanoalumina (Figure 5B).

Studies on the effects of nanoalumina on tight junction protein expression were completed by evaluation of permeability across the BBB. Injection with 1.25 mg/kg nanoalumina significantly elevated BBB permeability at day 3 post-injection and this effect was preserved for up to 30 days. Animals that received equimolar amounts of nanocarbon did not demonstrate disruption of the BBB.

Exposure to nanoalumina increases infarct volume induced by a permanent occlusion of the middle cerebral artery

The final series of experiments focused on the influence of nanoalumina on stroke development. Mice were injected with 1.25 mg/kg nanoalumina into the cerebral circulation, equimolar amount of nanocarbon, or a vehicle. In addition, selected mice were i.p. injected with alumina not converted to nanoparticles to avoid obstructing flow in the cerebral vessels. Three or 30 days after exposure to nanoalumina, stroke was induced by permanent occlusion of the left middle cerebral artery and stroke volume was calculated 24 h later. Exposure to nanoalumina significantly increased stroke volume. In contrast, treatment with nanocarbon or alumina of normal particle size did not affect stroke volume.

DISCUSSION

Unique characteristics of ENMs have raised concerns about their adverse effects on the environment and human health.^{3,4} ENMs can penetrate cell membranes and interfere with cellular organelles, executing potentially unexpected and/or unintentionally adverse effects. The present study has focused on the cerebrovascular toxicity of ENMs at the BBB level. In addition, because autophagy has been recognized as a general cell response to stress,¹⁶ we evaluated whether nanoalumina can act as an autophagy inducer in cells and animals. Induction of autophagy in brain endothelial cells and the brain endothelium was detected by elevated marker proteins, LC3 and p62. While p62 is a selective substrate for autophagy¹⁵, its expression is controlled by several regulatory mechanisms, including oxidative stress, Ras oncogene, or feedback regulation by NF- κ B. We indicated previously that nanoalumina can induce oxidative stress in cultured brain endothelial cells⁶. Importantly, increased p62 expression observed in the present study is consistent with elevated levels of LC3.

There are several cellular protein turnover mechanisms, including the ubiquitin-proteasome system (UPS) and autophagy-lysosomal system. The UPS is the primary route for degradation of short-lived or defective proteins and maintaining the amino acid pool in acute starvation. In contrast, autophagy is primarily responsible for degradation of long-lived proteins and maintaining amino acid pools in chronic starvation or stress. Although the experimental conditions chosen in the present study, such as a prolonged treatment with nanoalumina and focus on long-lived tight junction proteins, favor autophagic protein degradation, we cannot exclude a possible concurrent activation of the UPS system in our experimental models.

During autophagic processes, formation of autophagosomes and their fusion with lysosomes are the most critical steps leading to the formation of autophagolysosomes, at which stage proteins are degraded and amino acids are released.⁸ Recent reports have provided evidence that the mitochondrial membrane is important in the constitution of the double membrane structure of autophagosomes.¹⁷ Therefore, we examined the interaction of nanoalumina with

mitochondria, indicating that nanoalumina-induced dysfunctional mitochondria enter the autophagic degradation process.

Nanoalumina-induced disruption of mitochondrial function was also confirmed *in vivo* by a dramatic decrease in ATP levels in the cortex of exposed mice. These results are consistent with the malfunction of the respiratory chain, resulting in decreased mitochondrial potential and diminished ATP yield. Elimination of mitochondria via autophagy can be viewed as a cellular rescue mechanism aimed at maintaining cellular homeostasis. However, over-activated autophagy may lead to excessive protein degradation and autophagic cell death.¹⁸ Consistent with this notion, we indicated a substantial increase in protein degradation and enlarged infarct size as the results of nanoalumina insult.

The inability to fully remove dysfunctional mitochondria may stimulate release of cytochrome c from mitochondria, followed by apoptotic cell death.⁴⁸ Indeed, several genes (e.g., Bcl2l1, Bid, and Fas) that are directly involved in the stimulation of apoptosis were upregulated in the brain of nanoalumina-treated mice. However, exposure to nanoalumina also induced genes that regulate cytokine production and the MAPK signaling pathway, indicating that nanoalumina-induced cerebrovascular toxicity is a complex process, with autophagy being only one of several factors.

Exposure to nanoalumina also decreased expression of tight junction proteins, occludin and claudin-5, which are involved in maintaining the integrity of the BBB. Such an effect may be executed by several different mechanisms ranging from activation of signaling pathways, such as Rho and Ras-MAPK,¹⁹ and activation of proteolytic activity of matrix metalloproteinases and proteasome.¹⁵ The results of the PCR array, which identified stimulation of the genes of the MAPK pathway, are consistent with our previous finding on the importance of this signaling pathway in regulation of tight junction protein expression.^{15,19,20} Pretreatment with Wortmannin partially protected against nanoalumina-induced alterations of claudin-5 but not occludin, suggesting a potential involvement of autophagy in the regulation of selected tight junction proteins. While this is a novel observation, these results should be interpreted with caution because Wortmannin is not a selective inhibitor of autophagy and its effects include inhibition of PI-3 kinase.²¹

Persistent accumulation of nanoalumina in the CNS may predispose the brain to the development of acute and/or chronic neurological disorder. Indeed, we indicated that exposure to nanoalumina may aggravate the outcome of stroke, a disorder that has very strong neurovascular and neuroinflammatory components. We postulate that nanoalumina-induced oxidative stress, proinflammatory responses,^{5,6} and also enhancement of autophagic activity in exposed brains may contribute to stroke development. Importantly, our data indicate that even transient exposure to nanoalumina can have long-lasting effects with activated autophagy, disruption of tight junction proteins, and increased BBB permeability for as long as 30 days post-initial and transient exposure.

Nanocarbon was used in the present study at equimolar concentrations of nanoalumina to control for potential non-specific effects of nanoparticles. While nanoalumina was highly toxic to cells and brains of exposed animals, nanocarbon induced markedly lower toxic effects. These results clearly demonstrate that not only the size of ENMs but also their chemical and physical properties, surface modifications, electrical charges, and other characteristics can influence biological activity and toxicity of nanoparticles. Therefore, it is critical to investigate nanotoxicity of ENMs individually to establish appropriate recommendations regarding handling strategies and risk assessment.¹

In summary, the current study demonstrates that nanoalumina can accumulate in brain endothelial cells and the brains of exposed animals, inducing neurovascular damage and

activation of autophagy. Importantly, our results indicate that exposure to nanoalumina may increase vulnerability of the CNS and worsen the outcome of neurovascular disorders such as stroke.

Acknowledgments

Support: MH63022, MH072567, DA027569, ES07380, DA027569, and postdoctoral fellowship for Dr. Lei Chen from the American Heart Association.

References

1. Service RF. Nanotoxicology. Nanotechnology grows up. *Science*. 2004; 304:1732–4. [PubMed: 15205504]
2. Kagan VE, Bayir H, Shvedova AA. Nanomedicine and nanotoxicology: Two sides of the same coin. *Nanomedicine*. 2005; 1:313–6. [PubMed: 17292104]
3. Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science*. 2006; 311:622–7. [PubMed: 16456071]
4. Bondy SC. The neurotoxicity of environmental aluminum is still an issue. *Neurotoxicology*. 2010; 31:575–81. [PubMed: 20553758]
5. Oesterling E, Chopra N, Gavalas V, Arzuaga X, Lim EJ, Sultana R, et al. Alumina nanoparticles induce expression of endothelial cell adhesion molecules. *Toxicol Lett*. 2008; 178:160–6. [PubMed: 18456438]
6. Chen L, Yokel RA, Hennig B, Toborek M. Manufactured aluminum oxide nanoparticles decrease expression of tight junction proteins in brain vasculature. *J Neuroimmune Pharmacol*. 2008; 3:286–95. [PubMed: 18830698]
7. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron*. 2008; 57:178–201. [PubMed: 18215617]
8. Cuervo AM. Autophagy: In sickness and in health. *Trends Cell Biol*. 2004; 14:70–7. [PubMed: 15102438]
9. Thurn KT, Paunesku T, Wu A, Brown EM, Lai B, Vogt S, et al. Labeling tio2 nanoparticles with dyes for optical fluorescence microscopy and determination of tio2-DNA nanoconjugate stability. *Small*. 2009; 5:1318–25. [PubMed: 19242946]
10. Thurn KT, Arora H, Paunesku T, Wu A, Brown EM, Doty C, et al. Endocytosis of titanium dioxide nanoparticles in prostate cancer pc-3m cells. *Nanomedicine*. 2011; 7:123–30. [PubMed: 20887814]
11. Vazquez CL, Colombo MI. Assays to assess autophagy induction and fusion of autophagic vacuoles with a degradative compartment, using monodansylcadaverine (mdc) and dq-bsa. *Methods Enzymol*. 2009; 452:85–95. [PubMed: 19200877]
12. McLeland CB, Rodriguez J, Stern ST. Autophagy monitoring assay: Qualitative analysis of map lc3-i to ii conversion by immunoblot. *Methods Mol Biol*. 2011; 697:199–206. [PubMed: 21116969]
13. Chen L, Swartz KR, Toborek M. Vessel microport technique for applications in cerebrovascular research. *J Neurosci Res*. 2009; 87:1718–27. [PubMed: 19115415]
14. Neun BW, Stern ST. Monitoring lysosomal activity in nanoparticle-treated cells. *Methods Mol Biol*. 2011; 697:207–12. [PubMed: 21116970]
15. Komatsu M, Ichimura Y. Physiological significance of selective degradation of p62 by autophagy. *FEBS Lett*. 2010; 584:1374–1378. [PubMed: 20153326]
16. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008; 132:27–42. [PubMed: 18191218]
17. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell*. 2010; 141:656–667. [PubMed: 20478256]
18. Nixon RA. Autophagy in neurodegenerative disease: Friend, foe or turncoat? *Trends Neurosci*. 2006; 29:528–535. [PubMed: 16859759]

19. Zhong Y, Hennig B, Toborek M. Intact lipid rafts regulate hiv-1 tat protein-induced activation of the rho signaling and upregulation of p-glycoprotein in brain endothelial cells. *J Cereb Blood Flow Metab.* 2010; 30:522–533. [PubMed: 19794400]
20. Andras IE, Pu H, Tian J, Deli MA, Nath A, Hennig B, et al. Signaling mechanisms of hiv-1 tat-induced alterations of claudin-5 expression in brain endothelial cells. *J Cereb Blood Flow Metab.* 2005; 25:1159–1170. [PubMed: 15815581]
21. Blommaert EF, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and ly294002 inhibit autophagy in isolated rat hepatocytes. *Eur J Biochem.* 1997; 243:240–246. [PubMed: 9030745]

Figure 1. Nanoalumina enters HCMEC and accumulates in the brain

A. Cultured HCMEC were exposed to 1 µg/mL ARS-labeled nanoalumina (red staining) for the indicated time. The cells were co-stained with MitoTracker Green (green stain) to visualize mitochondria. ARS-nanoalumina was visible inside HCMEC 2 h post-exposure (arrows) and as aggregates (arrows) in the proximity to clustered mitochondria after a 12 h exposure. **B.** Localization of nanoalumina to cerebral vessels 1 h post ARS-nanoalumina administration. Cerebral vessels and astrocytes were visualized (green) using antibodies against Factor VIII and GFAP, respectively. ARS-nanoalumina localizes to the cerebral vessels (left panel, arrows) and is present in astrocytes surrounding the vessel (arrows). **C.** Nanoalumina is detectable in the brains for up to 1 week post-injection. ARS-nanoalumina exhibits a diffuse pattern in the brain parenchyma both 24 h and 1 week post-administration (arrows).

Figure 2. Nanoalumina treatment induces mitochondrial disruption and autophagic activity

A. HCMEC were treated with the indicated concentrations of nanoalumina for 6 h, followed by staining with MitoTracker Red (mitochondrial marker; red staining) and monodansylcadaverine (MDC, a marker for autophagic vacuoles, blue staining). The merged images demonstrate co-localization of clustered mitochondria and autophagic vesicles (arrows). Selected cultures were pretreated with Wortmannin (100 nM for 1h, PI3K inhibitor and inhibitor of autophagy) or exposed to nanocarbon (1.2 µg/mL for 6 h) or chloroquine (50 µM for 1h; positive control to induce autophagy). **B.** Nanoalumina-induced formation of autophagolysosome in HCMEC. Enlarged images of cultures exposed to 1 µg/mL nanoalumina for 6 h from (A) depict co-localization of altered mitochondria (stained with MitoTracker Red), lysosomes (stained with LysoTracker Green), and autophagic vacuoles (stained with MDC). Arrows in merged images indicate clustered mitochondria inside autophagolysosome. **C.** Mice were administered nanoalumina (1.25 mg/kg) or vehicle via the carotid artery and ATP levels in the cortex were assessed 24 h post-injection. Data are mean ± SEM, n=3-5. ***Significantly different as compared to vehicle treated animals at $p < 0.001$.

Figure 3. Nanoalumina induces expression of autophagy-related genes in the brains of exposed mice

A. Mice were exposed to nanoalumina as in Figure 1B for 24 h. Autophagy-related genes were analyzed using mouse Autophagy PCR Array in the whole brain extracts. **B.** Expression of the gene encoding for LC3 was confirmed by real-time PCR. Results are the mean $\pm$ SEM, n=4. *Significantly different as compared to vehicle treated mice at **, $p < 0.01$; or ***, $p < 0.001$.

Figure 4. Nanoalumina induces expression of autophagy-related proteins in HCMEC and the brains of exposed mice

A. HCMEC were exposed to the indicated concentrations of nanoalumina or 1.2 µg/mL nanocarbon (nC) for 24 h. Autophagy marker proteins, LC3 and p62, were then analyzed by immunoblotting. **B.** Mice were exposed to nanoalumina 1.25 mg/kg via the carotid artery. LC3 and p62 proteins were analyzed 1, 3, and 30 days post-nanoalumina exposure. The blots in (A) and (B) are representative images from four independent experiments. Results

are the mean $\pm$ SEM. *Significantly different as compared to control (vehicle treated cells or mice) at **, $p < 0.01$; or ***, $p < 0.001$. [†]Data in cells exposed to 1.2 $\mu\text{g/mL}$ nC are significantly different as compared to those exposed to 10 $\mu\text{g/mL}$ nanoalumina at $p < 0.05$. **C.** Brain sections were stained for Factor VIII (marker of the endothelium), GFAP (marker of astrocytes), and LC3 protein (red staining) 3 and 30 days post-nanoalumina injection. At day 3, LC3 was associated with the cerebral vessels. At day 30, LC3 presence was detected in the brain parenchyma.

A

B

Figure 5. Nanoalumina elevates protein degradation and induces disruption of tight junction proteins

A. HCMEC were exposed to increasing concentrations of nanoalumina or 1.2 $\mu\text{g/mL}$ nanocarbon for 24 h. Then, cells were loaded with DQ-BSA as the exogenous substrate for protein degradation, co-stained with LysoTracker Green to detect lysosomes and autophagolysosomes (green staining), and with DAPI to visualize nuclei. Arrows indicate fluorescent products of DQ-BSA proteolysis. **B.** HCMEC were treated with nanoalumina as in **A**. Selected cultures were pre-treated with Wortmannin (100 nM) for 1 h, followed by exposure to 10 $\mu\text{g/mL}$ nanoalumina for 24 h. Expression of tight junction proteins claudin-5 and occludin was then evaluated by immunoblotting. Results are the mean $\pm$ SEM.

***Significantly different as compared to vehicle treated cells at $p < 0.001$. †Data in cells pretreated with Wortmannin and exposed to 10 $\mu\text{g/mL}$ nanoalumina are significantly different as compared to those exposed to 10 $\mu\text{g/mL}$ nanoalumina alone at $p < 0.05$. **C.** Mice were injected with nanoalumina (1.25 mg/kg) as in Figure 1B and levels of claudin-5 and occludin were assessed 1, 3, 5, and 30 days post-injection. In addition, selected mice were injected with the equimolar levels of nanocarbon (nC) and claudin-5 and occludin levels were analyzed 30 days post-administration. *Significantly different as compared to vehicle-injected mice at **, $p < 0.01$; or ***, $p < 0.001$. †Data in mice exposed to nC for 30 days are significantly different as compared to those exposed to nanoalumina for 30 days at $p < 0.05$.

D. Mice were treated with nanoalumina (nAl) or nanocarbon (nC) as in **C**, followed by the assessment of the BBB integrity 3 and 30 days post-administration. Results are the mean $\pm$ SEM. *Significantly different as compared to vehicle or nC-injected mice at the corresponding time after the injection at *, $p < 0.05$; or ***, $p < 0.001$.

Figure 6. Exposure to nanoalumina exacerbates ischemic brain damage

Mice were injected into cerebral circulation with vehicle, nanoalumina (nAl) as in Figure 1B, or equimolar amounts of nanocarbon (nC). In addition, selected mice were injected i.p. with equimolar amounts of alumina (Al) of normal particle size to avoid obstructing flow in cerebral vessels. Three or 30 days post-nanoalumina treatment, the animals were subjected to permanent occlusion of the left middle cerebral artery. Infarct volume was assessed 24 h post-stroke induction. Results are the mean $\pm$ SEM. **Significantly different when compared to vehicle-injected mice at $p < 0.01$.