Crucial Role of P2X₇ Receptor in Regulating Exocytosis of Single-Walled Carbon Nanotubes in Macrophages

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Exocytosis of single-walled carbon nanotubes (SWCNTs) determines therapeutic efficiency and toxicity of nanoproducts but its underlying mechanism remains elusive. In this study, it is found that the exocytosis mechanism of SWCNTs is mediated mainly through the activation of P2X₇ receptor (P2X₇R), an ATP-gated membrane receptor highly expressed in macrophages. Inhibition of P2X₇R signaling by either a specific inhibitor (oxidized ATP) or small interfering RNA targeting P2X₇R largely prevents the exocytosis of SWCNTs from Raw264.7 cells, resulting in significant accumulation of SWCNTs within cells. In contrast, activation of P2X₇R with ATP promotes exocytosis of SWCNTs. Specifically, it is elucidated that internalized SWCNTs are accumulated in lysosomes and induce transitional release of ATP into extracellular space, which further activates P2X₇R, leading to the influx of calcium ions, phosphorylation of protein kinase C, ERK1/2, p38, and JNK, as well as alkalization of lysosomes. SWCNTs exposure also induces microtubules reorganization that facilitates the secretion of SWCNTs-containing lysosomes. It is also found that P2X₇R simultaneously mediates secretion of IL-1 β from Raw264.7 cells during the process of SWCNTs exocytosis. The combined data reveals that P2X₇R-mediated pathway is the predominant molecular mechanism for exocytosis of SWCNTs in Raw264.7 cells. Moreover, SWCNT-induced inflammation is closely coupled with the exocytosis of SWCNTs through P2X₇R.

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1. Introduction

Single-walled carbon nanotubes (SWCNTs), due to their unique physicochemical properties including high specific surface area, strength, conductivity, magnetic susceptibility, and catalytic activity, became one of the most shining stars among nanomaterials and were extensively explored for applications in biomedical fields, such as drug delivery, bioimaging, and so on.^[1–3] For an improved efficacy of nanosystems, much efforts have been made to enhance uptake and inhibit removal of nanocarriers by cells since the sustained therapeutic effect inside cells relies not only on the cellular uptake of nanocarriers but also on their subsequent long-term retention in the cells. Alternatively, from a toxicological point view, the uptake and retention dictate the internal exposure concentration inside cells, and thus the toxicity of the exposing

nanoparticles. Therefore, an in-depth understanding on the mechanisms of CNTs cellular uptake and exocytosis would facilitate the design of nanoproducts with not only better functionality and targeted therapeutic efficiency but also with controlled toxicity.

The cellular uptake of CNTs has been demonstrated by a variety of studies and the corresponding mechanisms were revealed.^[4–7] Similarly, exocytosis of nanoparticles is not uncommon, nanoparticles including peptide/protein-coated gold nanoparticles,^[8,9] silica nanoparticles,^[10] polylactico-glycolic acid nanoparticles,^[11] D-penicillamine coated quantum dots,^[12] and DNA-wrapped SWCNTs^[13,14] were reported to be repelled by various cell types. Normally, there are three pathways for the exocytosis of nanoparticles including lysosome secretion, vesicle-related secretion, and nonvesicle-related secretion.^[15] Nanoparticles, such as quantum dots, glycol chitosan, and superparamagnetic nanoparticles were mostly located in endosomes/lysosomes after internalization via endocytosis or phagocytosis pathways.^[12,16,17] In addition, the lysosomal accumulations of carbon nanoparticles including fullerene (C₆₀), single-walled carbon nanohorn, and nanotubes have also been documented.^[18–20] Therefore, it is reasonable to hypothesize that the SWCNTs exocytosis might be related to the regulation of lysosomal secretion. However, up to date, the underlying mechanisms are largely unknown.

Lysosomes are membrane-bound lytic organelles that were thought to be the endpoint of the endocytosis pathway to which protein and extracellular particles are delivered.^[21] However, they are now recognized to be dynamic organelles capable of fusing with a variety of targets such as autophagosomes and are also secretory organelles.^[22] Purinergic receptor P2X₇ (P2X₇R) is an extracellular adenosine triphosphate (ATP)-gated ion channel highly expressed on the plasma membrane of immune cells including T cells, dendritic cells, and monocytes/macrophages.^[23–25] The role of P2X₇R in mediating the inflammatory response was well characterized and the activation of P2X₇R was observed in cells in response to bacteria and parasite infections as well as silica and TiO₂ nanoparticles.^[26–30] Upon stimulation, P2X₇R rapidly induces NOD-like receptor protein (NLRP3)/caspase-1 inflammasome signaling complexes that drive the secretion of IL-1 β .^[31] However, P2X₇R was also recently shown to mediate the release of autophagolysosomes from MG6 cells^[32] and stimulate the exocytosis of secretory lysosomes in macrophages,^[33,34] suggesting a pleiotropic effect of P2X₇R. These observations provide us an intriguing clue on the potential mechanism of the exocytosis of SWCNTs involving the activation of P2X₇R.

Nanoparticles entered bodies tend to be recognized and taken up by macrophages of the mononuclear phagocytic system such as liver and spleen, where nanoparticles are accumulated and the chances of unintended acute or chronic toxicity are increased.^[15] Therefore, it is crucial to study the exocytosis of CNTs from macrophages. Given the capability of P2X₇R in regulating the fate of lysosomes, this study was designed to explore the role of P2X₇R in SWCNTs exocytosis in macrophages. By doing this, a macrophage cell line, Raw264.7, was employed and we provided convincing

evidences showing a novel role of P2X₇R played in mediating the exocytosis of SWCNTs, beside inflammation. More specifically, exposed SWCNTs are accumulated in lysosomes and increase the level of extracellular ATP, which activates a series of P2X₇R-mediated signaling pathways, leading to the rearrangement of microtubules and the release of SWCNTs-containing lysosomes.

2. Results and Discussion

2.1. Characterization of SWCNTs

Acid-oxidization of pristine SWCNTs greatly facilitates the dispersion of SWCNTs, removes residual metallic catalyst impurities, and shortens SWCNTs. The resulting SWCNTs compatible with aqueous environments have been used for the development of tumor-targetable multifunctional SWCNTs platforms^[35] and hold enormous potential for future uses. In this study, we prepared the SWCNTs by acid-oxidation method, which enables insertion of carboxyl and hydroxyl groups around the sidewalls and at the tips of the SWCNTs, which makes the tubes well dispersed in aqueous medium for days (**Figure 1A**). Transmission electron microscopy (TEM) characterization revealed that SWCNTs retained the structural integrity of carbon nanotubes (**Figure 1B**). Fourier transform infrared spectroscopy (FT-IR) analysis indicated the presence of carboxyl groups ($\nu = 1635\text{ cm}^{-1}$, -COOH) and hydroxyl group ($\nu = 1397\text{ cm}^{-1}$, -OH) on their surface (**Figure 1C**). Raman spectrum analysis showed typical G-band (1594 cm^{-1}) and D-band (1347 cm^{-1}) (**Figure 1D**), which is consistent with our previous study.^[20] In addition, the hydrodynamic diameters of SWCNTs ($20\text{ }\mu\text{g mL}^{-1}$) were 90.3, 1368, and 127.5 nm in water, serum-free medium (SFM), and serum-supplemented medium (SSM), respectively. The corresponding zeta potentials were -47.5 , -22.5 , and -8.3 mV , respectively (**Table 1**). Hydrodynamic diameters and surface charges of nanomaterials changed in serum-supplemented medium and serum-free medium compared to water because in serum free medium, the ions would be neutralizing the surface charge of SWCNTs and causes aggregation,^[36] but in serum medium, the serum protein would facilitate the dispersion of SWCNTs, resulting lower hydrodynamic size, compared with serum free medium, but still larger than that in water.

2.2. Localization of SWCNTs within Lysosomes in Macrophages and SWCNT-Induced ATP Release

Biological effects of nanoparticles are closely related to their cellular transportation and localization inside cells. To track the distribution of SWCNTs inside cells, we first looked into ultrathin sections of cells. TEM examination on macrophages showed the presence of a large amount of needle-like SWCNTs in lysosomes/endosomes (**Figure 2A**). Moreover, we further tracked the specific location of SWCNTs within living cells by labeling SWCNTs with fluorescent Alexa Fluor 488 (SWCNT-AF488). As shown in **Figure 2B**, the

Figure 1. Characterization of SWCNTs. A) Well dispersed SWCNTs in water in different concentrations (5, 10, 50, 200, and 1000 $\mu\text{g mL}^{-1}$). The suspensions can be stable for days. B) TEM image of SWCNTs. C) FT-IR spectroscopy of SWCNTs. D) Raman spectroscopy of SWCNTs.

green fluorescence from SWCNT-AF488 well overlapped with the red signal from LysoTracker Red, a fluorescent probe targeting lysosomes, indicating that SWCNTs were mostly accumulated in lysosomes.

ATP is often found in small concentrations (1×10^{-9} – 50×10^{-9} M)^[37] in extracellular space under physiological conditions and as an activator of P2X₇ receptor, extracellular ATP (eATP) is of significance in mediating a series of molecular events including exocytosis of lysosomes.^[31] Since activated or stressed cells were shown to release ATP,^[29] we investigated whether SWCNTs act through ATP leakage to trigger exocytosis process. Luciferase ATP assays revealed that SWCNTs triggered the release of ATP from cells, which peaks at 10 min (Figure 2C). Since eATP, in turn, can activate P2X₇R in an autocrine or paracrine manner to release ATP from cells, forming a positive feedback loop, the accumulation of eATP is the function of ATP release by cells and ATP hydrolysis by ectonucleotidases, the P2X₇R antagonist oxidized ATP (oATP) was used to limit the ATP release. As displayed in Figure 2C, pretreatment of cells with oATP could break the feedback loop, suppressed the level of eATP to that of background levels. Hence, ATP release induced by SWCNTs is transient process. Importantly, cell death measured by water-soluble tetrazolium-1 (WST-1) was minimal at concentrations $< 20 \mu\text{g mL}^{-1}$ (Figure S1, Supporting Information), suggesting that ATP release is not the consequence of any macrophage death.

Table 1. Hydrodynamic size (HDS, nm) and ZP (mV) of SWCNTs in water, SFM, and SSM.

SWCNTs	Water	SFM	SSM
HDS	90.3 ± 3.5	1368 ± 78	127.5 ± 6.3
ZP	-47.5 ± 1.2	-22.5 ± 0.8	-8.3 ± 0.1

2.3. SWCNTs Induced Ca^{2+} Influx through P2X₇R

Upon ATP stimulation, the fast and reversible opening of P2X₇R channel could lead to calcium ion influx in cells, which has also been documented in macrophages in response to stimulations.^[38–40] As a second messenger, the rise of intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) appears to be important in both normal physiology as well as pathophysiological processes in a variety of cell types including T cells, smooth muscle cell, and macrophages.^[41–43] There are compelling evidences that the increased cytosolic Ca^{2+} level is required for exocytosis of lysosomes.^[44,45] We thus determined $[\text{Ca}^{2+}]_i$ in macrophages. Meanwhile, in order to determine whether P2X₇R is responsible for calcium ion influx or not, we knocked down the expression of P2X₇R in macrophages using P2X₇R-specific small interfering RNA (P2X₇R siRNA). The siRNA treatment had no significant effect on cell viability (Figure S2, Supporting Information) but decreased the level of P2X₇R mRNA by 84% (Figure 3A) and 88% at the protein level (Figure 3B). These results confirmed that P2X₇R siRNA was efficient for P2X₇R gene silencing and the resulting cells were used for following experiments. In normal cells (no P2X₇R knock-down), as shown in Figure 3C, SWCNTs exposure induced robust increase of F340/F380 ratios in a concentration-dependent manner, indicating that SWCNTs are capable of inducing calcium ion influx, which leads to elevated $[\text{Ca}^{2+}]_i$. The normal cells and P2X₇R siRNA cells had similar $[\text{Ca}^{2+}]_i$ in control group (Figure 3D). However, in P2X₇R siRNA cells, SWCNTs-induced elevation of $[\text{Ca}^{2+}]_i$ was significantly suppressed, compared to that in normal cells treated with the same concentration of SWCNTs ($10 \mu\text{g mL}^{-1}$) (Figure 3D), indicating the pivotal role of P2X₇R in SWCNTs-induced Ca^{2+} influx. These

Figure 2. A) TEM imaging of macrophages (Raw264.7) after incubation with 0 (left) and 5 µg mL⁻¹ (right) SWCNTs for 24 h. Red arrows indicate lysosome/endosomes membrane and blue arrows for SWCNTs. B) Laser confocal imaging of cells incubated with 5 µg mL⁻¹ SWCNT-AF488 or unlabeled SWCNTs for 24 h. The cells in all groups were then labeled with 200 × 10⁻⁹ M LysoTracker Red for 30 min and observed using a laser confocal scanning microscope. The control cells (no treatment, a–d) showed lysosomes labeling only (white arrows). When cells were exposed to SWCNT-AF488 (e–h), SWCNTs were visualized in cells as green color (pink arrows) and colocalized with lysosomes. Cells treated with unlabeled SWCNTs (i–l) had lysosomal labeling only. C) Extracellular ATP determination in cell culture supernatant by luciferase assay. Cells were incubated with 0 (control), 2, and 5 µg mL⁻¹ SWCNTs for indicated time. The cells were also pretreated with P2X₇R antagonist (oATP) for 1 h and then exposed to 5 µg mL⁻¹ SWCNTs to reveal the role of P2X₇R. Data are representative of three independent experiments and are expressed as mean values ± S.D.

data suggest that SWCNTs-induced elevation of [Ca²⁺]_i is at least in part mediated by P2X₇R. The reason [Ca²⁺]_i did not return to the control level might be that there were still some P2X₇R expression left after knocking down (Figure 3A,B), or there are other pathways induced by SWCNTs, resulting in elevation of [Ca²⁺]_i.

2.4. SWCNTs-Induced Activation of Protein Kinase C (PKC) and mitogen-activated protein kinases (MAPK) Pathways was Mediated through P2X₇R

Intracellular calcium ion mediates a number of signaling transductions including PKC pathway, which might evoked the secretion of lysosomes via mediating protein phosphorylation cascades such as the down-stream mitogen-activated protein (MAP) kinases cascade.^[46–48] Ca²⁺-mediated phosphorylation of PKC was regarded as a key triggering step in the process of secretion of lysosome.^[46,49] To determine whether SWCNTs are able to activate the above mentioned pathways, Western-blot analyses were performed to detect the phosphorylation of PKC, extracellular regulated protein kinases 1/2 (ERK1/2), p38, and jun N-terminal kinase (JNK)

after exposing cells to SWCNTs. As shown in **Figure 4A**, the phosphorylation of PKC, ERK1/2, p38, and JNK was induced by SWCNTs in a concentration-dependent manner. Next, to investigate the role of P2X₇R played in the activation of these pathways, we knocked down the gene expression of P2X₇R in macrophages using siRNA. When we exposed the P2X₇R knocked-down cells to SWCNTs, the phosphorylation levels of these proteins almost returned to that of control group, except for the ERK1/2, the phosphorylation level of which was only partially alleviated (Figure 4B). Combined data strongly suggest that SWCNTs induced the phosphorylation of PKC and MAPKs by activating P2X₇R. The result is in agreement with the finding that upon P2X₇R activation, PKC was activated after binding with calcium ion and translocated to the basolateral membrane.^[50]

2.5. SWCNTs Induced pH Elevation, Microtubules Reorganization, and P2X₇R-Mediated Release of Lysosomes and SWCNTs

The secretion of lysosomes is usually accompanied with pH elevation in lysosomes and rearrangement

Figure 3. P2X₇R silencing of Raw264.7 cells and P2X₇R-mediated influx of calcium ions. A) Relative mRNA level of P2X₇R gene expression after P2X₇R siRNA treatment, compared with normal cells. B) Relative P2X₇R level after P2X₇R siRNA treatment, compared with control. C) F340/F380 ratio change over time in cells treated with different concentrations of SWCNTs. Cells were loaded with 5×10^{-6} M Fura-2AM and then treated with SWCNTs or ATP (2×10^{-3} M), followed by immediate time lapse fluorescence analyses. D) F340/F380 ratio change in cells with (designed as P2X₇R siRNA) or without P2X₇R siRNA treatment (Normal cells) and then subjected to $10 \mu\text{g mL}^{-1}$ SWCNTs exposure. ATP treatment was set as a positive control. Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. **: significant difference between groups, $p < 0.01$.

of microtubules.^[32,46] Macrophage lysosomes are usually maintained at acidic conditions ($\text{pH} \approx 4.5$). However, lysosomal alkalization could occur in response to extracellular stimuli.^[51] The pH-elevated lysosomes have less stability in cells and a slight elevation in lysosomal pH was sufficient to trigger secretion of lysosomes.^[52] We measured the pH change of macrophage lysosomes by using two lysosome-targeting probes, i.e., lysosensor DND-189 and lysosensor DND-153. The former can generate lower fluorescence intensity at a neutral pH than at an acidic pH, whereas the fluorescence of the latter undergoes opposite change upon pH alteration. Since the fluorescence changes of these two probes are in opposite directions, the possibility that the fluorescence change is due to the interference of SWCNTs with probes can be reasonably ruled out. As shown in **Figure 5A** (up-panel), B, DND-189 stained cells in ATP and SWCNTs treatment (1 and $2 \mu\text{g mL}^{-1}$) groups exhibited much lower fluorescence intensity in their cytoplasm than control cells did. In contrast, the same treatments lead to increased fluorescence intensities when cells were stained with DND-153 (**Figure 5A** (down-panel), C). The data demonstrate that SWCNTs cause pH elevation of lysosomes. The elevated lysosome pH may be due to P2X₇R-induced Ca^{2+} influx and PKC activation, which promote the proton release of lysosomes into cytoplasm and impair the ATP-driven proton pump on lysosomal membrane.^[32]

When lysosome exocytosis takes place, microtubules have to undergo a series of reorganization, changing from randomly directed bundles to radial extension towards plasma membrane, so that the lysosomes can move along microtubules for secretion.^[53,54] We examined the organization pattern of microtubules in cells received SWCNTs exposure by staining cells with fluorescence-labeled anti- β -tubulin antibody. By examining different areas of cell culture, we consistently found that the bundles of microtubules were loosely and randomly distributed in untreated cells, but in SWCNTs and ATP treated cells, the microtubules bundles were extended from cell nucleus toward the plasma membrane (typical images were shown in **Figure 5D**), suggesting that microtubules reorganization was practically occurring during SWCNTs exposure to provide orbits for the exocytosis of lysosomes, which was evident by measuring the marker protein of lysosomes, cathepsin D (31 kD, CTSD),^[55,56] in the supernatant after SWCNT exposure. The results showed that while ATP treatment increased the level of CTSD in supernatant significantly, SWCNTs elicited the secretion of CTSD in a concentration- and time-dependent manner, as shown in **Figure 5E,F**, respectively, indicating that lysosomes were indeed secreted by cells. Simultaneously, we measured the change of SWCNT amount in cell culture supernatants by using visible–near-infrared (vis–NIR) spectroscopy to directly detect the exocytosis of SWCNTs from cells. The

Figure 4. Western blot analyses on the relative phosphorylation levels of PKC and MAPK pathway proteins (Treatment/Control, T/C) in A) normal cells and B) P2X₇R silenced cells with or without SWCNT exposure. ATP treatment was set as positive control. Lower panels: Typical Western blot gel image of target proteins and reference protein, β -actin. Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. Asterisk indicates significant difference from control at level *: $p < 0.05$, **: $p < 0.01$.

results showed that SWCNTs increased in the supernatants in a concentration- and time-dependent manner, along with the secretion of lysosomes (Figure 5E,F, lower panels). Importantly, silencing of P2X₇R dramatically blocked the secretion of lysosomes (Figure 5G) and the SWCNTs exocytosis significantly diminished as well (Figure 5G, lower panel). Taken together, these results suggest that SWCNTs can induce the pH elevation of lysosomes and reorganization of microtubules, facilitating the secretion of lysosomes via the activation of P2X₇R. Importantly, during the process of lysosomal exocytosis, the contents of lysosomes, SWCNTs, were expelled into extracellular space. In case of SWCNTs exposure, P2X₇R-mediated lysosomal secretion might serve as a way of cells repeling the SWCNTs to maintain homeostasis.

2.6. P2X₇R Activation/Inhibition Influenced Exocytosis of SWCNTs

To finally confirm the crucial role of P2X₇R in regulating exocytosis of SWCNTs, the agonist (ATP) and antagonist (oATP) were employed to activate and block P2X₇R, respectively. The agonist and antagonist themselves had no significant effect on cell viability (Figure S3, Supporting

Information). sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to quantify the amount of intracellular SWCNTs as described previously.^[57] However, the ATP/oATP treatments also raised the question as to whether the ATP/oATP treatments influence SWCNT uptake instead of exocytosis or not. To exclude the possibility, the cells were first exposed to SWCNTs for 5 h and allowed to take up equal amount of SWCNTs for all groups, then the exposure solution were removed and ATP or oATP was added for incubation for 1 h, followed by replacing exposure medium with fresh medium. Subsequently, we measured the intracellular and extracellular SWCNTs amount by SDS-PAGE method and UV-vis-NIR at 24 h, respectively. As shown in **Figure 6A** (up panel), the intracellular SWCNTs amount decreased with increasing ATP concentrations, while the extracellular SWCNTs amount increased (lower panel). Furthermore, oATP treatment dramatically increased the retention of SWCNTs inside cells (Figure 6B, up panel) and diminished the exocytosis in a concentration-dependent manner (Figure 6B, lower panel). Most importantly, knock-down of P2X₇R expression resulted in the similar effect as those of oATP and in these cells (P2X₇R silenced), ATP or oATP treatments made no difference at all (Figure 6C). These results strongly suggest that exocytosis of SWCNTs is mediated by P2X₇R, inhibition or gene knock-down of

Figure 5. SWCNTs induced pH elevation, microtubules reorganization, and secretion of lysosomes and SWCNTs. A) Laser confocal images of cells under different treatments and labeled with lysosensor DND-189 (upper panel) or lysosensor DND-153 (lower panel). B) Quantification of fluorescence in cells labeled with lysosensor DND-189 and DND-153 (C). D) Immunostaining of microtubules of cells under the treatment of SWCNTs and ATP. Blue fluorescence: Hoechst 33342 staining of cell nucleus; Green fluorescence: Alexa Fluor 488 labeled goat anti-mouse IgG (secondary antibody). Scale bar = 20 μm . Relative amount of CTSD (Treatment/Control, T/C) and SWCNTs quantification in supernatant after the cells treated with SWCNTs in different concentrations for E) 24 h or F) at 10 $\mu\text{g mL}^{-1}$ for various time, as determined by Western blot and UV-vis-NIR spectroscopy. Upper panel: relative amount of CTSD protein in supernatant; Middle panel: representative image of Western-blot gel for CTSD protein; Lower panel: UV-vis-NIR spectra of SWCNTs in the supernatant. G) Relative amount of CTSD and SWCNTs in supernatant after exposing SWCNTs (10 $\mu\text{g mL}^{-1}$) to normal cells (set as control) or P2X₇R silenced cells. In western blot experiment, ATP treatment group was set as the positive control. Asterisk indicates significant difference from control at level *: $p < 0.05$, **: $p < 0.01$.

Figure 6. Influence of P2X₇R activation/inhibition on the exocytosis of SWCNTs. Relative SWCNT amounts within cells (upper panel) and in supernatants (lower panel) under the treatment of different concentrations of A) ATP (Treatment/Control, T/C) or B) oATP, as determined by SDS-PAGE gel electrophoresis and UV-vis-NIR spectroscopy analysis, respectively. C) Relative intracellular SWCNTs amount in P2X₇R silenced cells against that of normal cells (set as control) and extracellular SWCNTs amount determined by UV-vis-NIR spectroscopy (lower panel). Middle panels: representative images of SWCNTs bands after SDS-PAGE gel electrophoresis; lower panels: typical UV-vis-NIR spectra of the supernatant of SWCNTs. Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. Asterisk indicates significant difference from control at level *: $p < 0.05$, **: $p < 0.01$.

which would result in decreased exocytosis of intracellular SWCNTs and higher retention of SWCNTs inside cells.

2.7. Secretion of Inflammatory Cytokine IL-1 β in Response to SWCNTs

In addition to regulating the secretion of lysosomes, P2X₇R is also often demonstrated to mediate interleukin (IL)-1 β release in macrophages.^[29,31] Carbon nanotubes can, through the activation of P2X₇R, induce the formation of NLRP3 inflammasomes to catalyze the maturation and secretion of IL-1 β .^[58] We therefore measured IL-1 β release in SWCNTs-exposed macrophages to further confirm the activation of P2X₇R. As shown in **Figure 7A,B**, SWCNTs exposure induced active release of IL-1 β in both concentration- and time-dependent manner. To substantiate the role of P2X₇R in SWCNTs-induced release of IL-1 β , we pretreated the cells with P2X₇R agonist (ATP) and antagonist (oATP) and then exposed cells to 5 $\mu\text{g mL}^{-1}$ SWCNTs. Results showed that pretreating cells with ATP significantly increased IL-1 β secretion of SWCNTs-exposed cells (**Figure 7C**), while inhibition of P2X₇R by oATP pretreatment had the opposite effect (**Figure 7D**). Both effects were concentration-dependent. Taken together, these results strongly suggest that P2X₇R is indeed activated by SWCNTs, leading to the release of IL-1 β .

Upon exposure, SWCNTs can be taken up, retained, or excreted from nucleated cells.^[59,60] In this study, we investigated the underlying mechanism of SWCNTs exocytosis in a macrophage/monocytic cell line, Raw264.7. Macrophages represent the first line of defense in the immune system by engulfing invaders and clear them.^[26,61] On the other hand, macrophages are also involved in inflammatory response and tissue fibrosis via the activation of P2X₇R and NLRP3/caspase-1 inflammasome.^[23,31,62] At inflammatory sites, eATP may act as a danger signal to activate P2X₇R in an autocrine/paracrine manner. Simultaneously, lines of evidences indicate that ATP binding of P2X₇R stimulates the release of various intracellular macromolecules via secretory lysosomes.^[33,63] Here, we, for the first time, report that beside inflammation, P2X₇R also plays a critical role in mediating SWCNTs exocytosis through secretion of lysosomes, as demonstrated by our data that specific antagonist of P2X₇R substantially inhibited exocytosis of SWCNTs, while agonist of P2X₇R strongly promoted the exocytosis of SWCNTs (**Figure 6A,B**). Furthermore, in P2X₇R silenced cells, the intracellular SWCNTs were significantly increased compared to normal cells, but no effect of antagonist and agonist on this change was observed in this type of cells (**Figure 6C**), indicating that antagonist and agonist lost the binding target due to the absence of P2X₇R and strongly verified the crucial role of P2X₇R in regulating SWCNTs exocytosis. We also demonstrated that upon SWCNT exposure, P2X₇R mediated the secretion of IL-1 β

Figure 7. P2X₇R-mediated IL-1 β release from Raw264.7 cells upon SWCNTs exposure. A) Levels of IL-1 β in supernatant after cells were treated with different concentrations of SWCNTs. B) Levels of IL-1 β in supernatant after cells were treated with SWCNTs for indicated times. C) SWCNTs (5 $\mu\text{g mL}^{-1}$) induced IL-1 β release in supernatant after cells received different concentrations of ATP pretreatment. D) SWCNTs (5 $\mu\text{g mL}^{-1}$) induced IL-1 β release in supernatant after cells received different concentrations of oATP pretreatment. Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. Asterisk indicates significant difference from control at level *: $p < 0.05$, **: $p < 0.01$.

(Figure 7). However, the concurrence of P2X₇R-triggered lysosome exocytosis and IL-1 β release does not necessarily suggest the same mechanism of the two processes. Indeed, it has been shown that removal of extracellular Ca^{2+} completely abrogates ATP-induced lysosome exocytosis from murine macrophages without affecting IL-1 β secretion.^[33] In addition, during P2X₇R activation, no significant redistribution of cytosolic IL-1 β into secretory lysosomes was shown by immunocytochemical analyses on intact murine macrophages.^[64] These evidences unequivocally suggest the dissociation of lysosome exocytosis from the non-classical IL-1 β secretion machinery regulated by P2X₇R.^[65] Therefore, this reported underlying mechanism for the SWCNT exocytosis is distinct from the one for CNT-induced IL-1 β inflammation,^[58] except for the involvement of the common receptor, P2X₇R. One of the differences between the mechanisms relies on the reorganization of microtubules (Figure 5D), which is required for lysosome secretion but unnecessary for IL-1 β release.

The natural ligand of P2X₇R is ATP, a molecule that is produced ubiquitously and presents in the cytoplasm of every cell. It is worth of note that SWCNTs-induced ATP release peaked at 10 min and then returned to baseline (Figure 2C), suggesting that SWCNTs-induced ATP release is transient in Raw264.7 cells. Similar results were reported by Kojima et al. by showing that ATP release was the highest at 25 min in murine Kupffer cells (resident macrophages) treated with silica nanoparticle.^[29] Nevertheless, in THP-1 cells, the ATP release reached peaks upon 2 h exposure of monosodium urate (MSU) crystal.^[66] We speculate that the difference may

be due to either the different chemical and physical properties of particles (long aspect ratio fibers for CNTs vs short needle-like crystal for MSU) or the cell models (differentiated macrophages vs undifferentiated monocytic cells) with different expression levels of P2X₇R and ecto-ATPase, which hydrolyzes extracellular nucleoside tri- and/or diphosphates. On the other hand, transient stimulation with agonist (such as ATP) opens a P2X₇R channel permeable to small cations, whereas sustained agonist stimulation leads to a pore state permeable to moieties of up to 900 Da, which is most commonly associated with consequent cell death, apoptosis or cytolysis.^[67] Therefore, the transitional ATP release as revealed by this study explained the fact that cells are viable (Figure S1, Supporting Information) while P2X₇ receptors are activated. One can envision that with increasing concentration of SWCNTs, eATP levels would reach a threshold level overwhelming hydrolysis capacity of ecto-ATPase, leading to prolonged P2X₇R stimulation and consequently cytotoxicity. Currently, the precise mechanism of SWCNT-induced ATP release is still unknown, while there are three possible ways: (1) SWCNTs moderately damage cell membrane, resulting in leakage of cellular content including ATP; (2) SWCNTs interact and inhibit the activity of ecto-ATPases, causing higher extracellular ATP levels left; (3) SWCNTs interact directly with P2X₇R, opening the channel of the receptor and subsequently result in release of intracellular ATP. The answer to this question would provide a better understanding of initial molecular events for the activation of P2X₇R, which warrants detailed investigation in the future.

Scheme 1. Proposed SWCNTs exocytosis mechanisms. Endocytosed SWCNTs induce ATP release from cells to extracellular space, which binds to P2X₇R resulting in calcium ion influx. Thereafter, PKC, through binding to calcium ion, is activated and evokes MAPK pathways, leading to elevated lysosome pH value. The alkalinized lysosomes are unstable in cells and move along reorganized microtubules to fuse with plasma membrane and ultimately are released from cells, along with which SWCNTs are exocytosed.

Due to its proinflammatory property, P2X₇R was shown to play an essential role in pathophysiology of some neurological and cardiovascular disorders as well as cancers.^[68] Accordingly, it was regarded as a potential therapeutic target stratifying treatments of these diseases.^[69] However, our findings suggest caution on the strategy of controlling CNT-induced inflammation via P2X₇R inhibition as it would lead to accumulation of CNTs, which might be detrimental to cells. Instead, it is preferable to target down-stream signaling steps of inflammation that are unrelated to lysosome secretion. For example, pannexin-1 is required for the development of inflammation in enteric neuron and inhibiting of which would prevent inflammation-induced neuron cell death.^[70] Besides, inhibitor of cathepsin B could disrupt the assembly of NLRP3 inflammasome and reduce traumatic brain injury.^[71] By these ways, one can reach the purpose of resolving the side-effects of CNTs without introducing new problems. The exocytosis mechanism of SWCNTs in macrophages revealed by this study would allow us to develop more clearable delivery nanosystems after drug delivery and facilitate a better design of safer SWCNT products with controlled retention in body.

3. Conclusions

In summary, we demonstrated with evidences that internalized SWCNTs were accumulated in lysosomes of Raw264.7 macrophages and simultaneously stimulated ATP release, resulting in the activation of P2X₇R. The opening of P2X₇R

allowed the influx of calcium ions that further induced the activation of PKC and MAPK signaling pathways, pH elevation in lysosomes, reorganization of microtubules, and ultimately the secretion of SWCNTs-containing lysosomes. Inhibition or activation of P2X₇R by oATP or ATP pretreatment could modulate the retention and the exocytosis of SWCNTs in a concentration-dependent manner. Furthermore, gene expression knock-down of P2X₇R largely abrogated the exocytosis of SWCNTs, as well as the effects of ATP and oATP on the exocytosis, strongly suggested a pivotal role P2X₇R played during the exocytosis of SWCNTs. In addition, SWCNTs exposure induced secretion of IL-1 β , which could be modulated by the P2X₇R activator and inhibitor, indicating that the mechanisms underlying SWCNTs exocytosis and IL-1 β secretion are closely coupled together through P2X₇R. A proposed mechanism underlying P2X₇R-induced exocytosis of SWCNTs in macrophages is illustrated in **Scheme 1**. These findings would provide a novel insight into the control of SWCNTs toxicity and therapeutic efficiency.

4. Experimental Section

Reagents and Materials: The murine macrophage cell line Raw264.7 Cells (ATCC: TIB-71) was acquired from American Type Culture Collection. All ingredients for the culture media were purchased from Gibco, Invitrogen (UK). SWCNTs (CNTs purity > 95%, SWCNT purity > 90%, ash < 1.5 wt%) synthesized by chemical vapor deposition method were originally obtained from Chengdu Organic Chemicals Co., Ltd (SiChuan, China) in high purity. The

detailed information can be found on the company website: <http://www.timesnano.com/>. Cathepsin D (CTSD) antibody, phospho-PKC antibody, rabbit mAbs against P2X₇R, phospho-p44/42 MAPK, phospho-p38 MAPK, phospho-JNK MAPK, and β -actin or goat anti-rabbit horseradish peroxidase (HRP) antibody were purchased from Cell Signaling Technology (USA). The Fura-2AM, LysoSensor DND-189, LysoSensor DND-153, LysoTracker Red DND-99, Alexa fluor 488 probes, and anti-mouse IgG Alexa Fluor 488 were purchased from Invitrogen (USA). ATP, oATP, Hoechst33342, anti- β -tubulin mouse mAb, and BSA were purchased from Sigma-Aldrich (USA). Mouse IL-1 β enzyme-linked immunosorbent assay (ELISA) set was purchased from BD Biosciences (USA).

Preparation of Single-Walled Carbon Nanotubes: Preparation of SWCNTs solution was performed according to the procedure described previously.^[72] In detail, as-produced SWCNTs (10 mg) were suspended in 40 mL of a 3:1 mixture of concentrated H₂SO₄/HNO₃ in a 200 mL flask and sonicated in a water bath (KQ-500DV, 40 kHz) for 24 h at 40–50 °C. The resultant suspension was then diluted with 200 mL deionized water and filtered through a membrane (pore size 0.22 μ m), followed by wash with 50 mL deionized water on the membrane. The single-walled carbon nanotubes were resuspended in sterilized deionized water at a concentration of 1 mg mL⁻¹ with brief sonication for 5 min (KQ-500DV, 40 kHz). The acid-functionalized SWCNTs suspension was black, well dispersed, and had neutral pH.

Characterization of SWCNTs: Prepared SWCNT suspension was diluted to 0.5 mg mL⁻¹ and precipitated onto a copper net and dried for imaging with a HitachiH-7500 transmission electron microscopy (TEM, Tokyo, Japan). In addition, the infra-red spectra of SWCNTs were collected by using a FT-IR spectrometer (JASCO, Inc., Easton, MD, USA). Zeta potential (ZP) and hydrodynamic size of SWCNTs were measured by Zetasizer Nano (Malvern Instruments, Malvern, UK). Raman measurement was performed via using Renishaw Raman spectroscopy (Wotton-under-Edge, UK) with excitation wave length at 532 nm.

Cell Culture and Exposure: Raw264.7 cells were cultured at 37 °C in fully humidified atmosphere containing 5% CO₂ in complete culture medium (cRPMI) consisting of RPMI-1640 and 10% heat deactivated fetal bovine serum (FBS) supplemented with 20 $\times$ 10⁻³ M L-glutamine and 100 U mL⁻¹ penicillin/streptomycin. For all experiments of SWCNTs quantification, cells were seeded in 6-well plates (Corning, USA) at a density of 5 $\times$ 10⁵ cells mL⁻¹ in cRPMI and allowed to attach for overnight. The stock solution of 1 mg mL⁻¹ SWCNTs was prepared by sonicating in a cold water bath (KQ-500DV, 40 kHz) for 5 min before usage. Thereafter, the stock solutions were diluted in cRPMI to desired concentrations for exposure. The conditions for all experiments were optimized. As a result, the concentrations of SWCNTs were set as 1, 2, 5, and 10 μ g mL⁻¹. For the experiment involving fluorescence probes, 10 μ g mL⁻¹ SWCNTs was not used to exclude the interference of SWCNTs on the measurements of fluorescence intensity, except for calcium experiment, which used fluorescence ratio as the endpoint. For SWCNTs quantification and protein analyses, higher concentrations of SWCNTs such as 2, 5, and 10 μ g mL⁻¹ were used. As to the timepoints, our previous data (not shown) suggested that the SWCNTs amount within cells reached highest after 5 h following by rapid decreasing of intracellular SWCNTs, therefore the 5 h was chosen for microtubule reorganization detection, assuming that the change (reorganization of microtubules for exocytosis)

would occur then. For other experiments, the 24 h was used as a common timepoint as specified.

Ultrathin Section of Macrophages for SWCNTs Examination: The distribution of SWCNTs in Raw264.7 cells was investigated by TEM. Cells (2 $\times$ 10⁶ mL⁻¹) exposed to 5 μ g mL⁻¹ SWCNTs for 24 h were collected, washed twice with phosphate buffer saline (PBS) and pelleted by centrifugation at 1500 rpm for 10 min at 4 °C. The cells were then prefixed with 2.5% glutaraldehyde in PBS for 10 min at room temperature and stored at 4 °C overnight. After that, cells were post-fixed with 1% osmium tetroxide at room temperature for 2 h, which was followed by dehydration and resin embedding. Ultrathin sections of macrophages were cut by using a Leica EMUC6 ultramicrotome (Wetzlar, Germany) and settled on 200-mesh carbon-coated copper grids. After staining with uranyl acetate and lead citrate, the samples were observed with a FEI TENAI Spirit TEM (Eindhoven, Noord-Brabant, The Netherlands) at 100 kV.

Labeling of SWCNTs for Tracking SWCNTs within Cells: To determine whether SWCNTs within macrophages were localized in lysosomes or not, part of above-mentioned SWCNTs was covalently labeled with an amine-containing fluorescent dye, Alexa Fluor 488, through (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride)/(N-hydroxysulfosuccinimide) mediation in 2-morpholinoethanesulfonic acid buffer, as described previously.^[73] The labeled SWCNTs (SWCNT-Alexa Fluor 488) were further purified and concentrated by centrifugation at 12 000 rpm in ultrafiltration tubes (Millipore, Germany) and then diluted to desired concentration. After 24 h exposure, cells were washed with PBS and then labeled with LysoTracker Red DND-99 (200 $\times$ 10⁻⁹) for 30 min according to the protocol provided by manufacturer (Invitrogen, USA). The colocalization of SWCNTs and lysosomes were performed via confocal laser scanning microscopy (Leica, Mannheim, Germany).

Extracellular ATP Measurements: Extracellular ATP concentration was measured by using Luciferase/Luciferin Reagent (Invitrogen, USA). Raw264.7 cells (5 $\times$ 10⁴ cells per well) were exposed to 0–5 μ g mL⁻¹ of SWCNTs in 200 μ L of cultural medium. To examine the effect of oATP (specific P2X₇R antagonist) on ATP concentration, cells were pretreated with 2 $\times$ 10⁻³ M oATP for 1 h and then oATP was replaced with SWCNTs exposure solution. After exposure, 20 μ L supernatant of culture medium were collected at indicated time points and immediately snap-frozen in liquid nitrogen for later ATP determination. The concentration of ATP was detected by measuring chemiluminescence on a Varioskan Flash microplate reader (Thermo, MA, USA) after addition of 200 μ L luciferin-luciferase reagent to the sample.

P2X₇ Receptor Silencing: Raw264.7 cells were transfected with siRNA to silence P2X₇ receptor expression. P2X₇R-targeting oligonucleotides were designed and generated from full-length mouse P2X₇R by Shanghai GeneChem Co., Ltd. (Shanghai, <http://www.genechem.com.cn>). After testing knockdown efficiencies, stem-loop DNA oligonucleotides were synthesized by Shanghai GeneChem Co. Ltd. (sense, 5'- CCG G GC GGA AAG AGC CTG TTA TCA GCT CGA GCT GAT AAC AGG CTC TTT CCG CTT TTT G-3'; antisense, 5'- AAT TCA AAA AGC GGA AAG AGC CTG TTA TCA GCT CGA GCT GAT AAC AGG CTC TTT CCG C-3') and cloned into the lentivirus-based RNAi vector GV248. A nontargeting stem-loop DNA GV248 vector was also generated for use as a negative control. Lentiviral particles were prepared according to protocol provided by GeneChem Co.

Ltd. Raw264.7 cells were then infected with P2X₇-RNAi-lentivirus or negative control virus in the presence of enhanced infection solution (GeneJikai, Shanghai, China) (multiplicity of infection = 100). Then, the cells were incubated with the mixed solution for 10 h at 37 °C in fully humidified atmosphere containing 5% CO₂. After the removal of infection solution, the cells were cultured in fresh medium for another two days. Following this, the cells were used for different experiments. Meanwhile, the cells were collected for quantitative reverse transcription polymerase chain reaction (QRT-PCR) or western blot.

P2X₇ Receptor Gene Expression and P2X₇ Receptor mRNA Transcription: The mRNA levels of P2X₇ receptors in cells after the knockdown of P2X₇R were measured by using real-time RT-PCR (LightCyclerC480, Roche, Germany). The primer sequences for quantitative PCR analyses are: P2X₇R (forward primer: 5'-AGACAGCCGATCTTCTGT-3'; Reverse primer: 5'-CTTGCCGTGGTGA-GAGTCAT-3'). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (forward primer: 5'-TGATGACATCAAGAAGGTGGTGAAG-3'; Reverse primer: 5'-CCTTGGAGGCCATGTAGGCCAT-3'). The comparative Ct method for relative quantification of gene expression ($\Delta\Delta C_t$ method) was used and gene expression levels of P2X₇ receptors were normalized to gene expression of GAPDH (as a housekeeping gene control) using the following equation: P2X₇R mRNA normalized expression = $2^{\Delta C_t (GAPDH) - \Delta C_t (P2X7R)}$.

Measurement of the Intracellular Free Ca²⁺ Concentration ([Ca²⁺]): Raw264.7 cells were cultivated on a 96-well plate for 24 h and then loaded with 5×10^{-6} M Fura-2AM at 37 °C for 30 min in cRPMI. Thereafter, the cells were rinsed with washing buffer (155×10^{-3} M NaCl, 4.5×10^{-3} M KCl, 1×10^{-3} M MgCl₂, 2×10^{-3} M CaCl₂, 10×10^{-6} M D-glucose, 5×10^{-6} M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and then exposed to SWCNTs. The plate was immediately measured with a Varioskan Flash microplate reader. The increase of intracellular [Ca²⁺], was expressed as the fluorescence intensity ratio measured at 340 and 380 nm excitation wavelength (F340/F380), as it is proportional to calcium ion concentrations. F340/F380 ratiometric measurement of emission at 510 nm was obtained every 54 s upon SWCNT exposure. The experiment was carried out in the dark.

SDS-PAGE and UV-vis-NIR for Quantification of SWCNTs in Cells and Supernatants: The same number of cells were seeded and treated with $10 \mu\text{g mL}^{-1}$ SWCNTs for 5 h and washed three times with PBS buffer to remove excess SWCNTs. The cells were then incubated with series concentrations of ATP or oATP for 1 h, followed by replacing exposure medium with fresh medium. After 24 h, same amount of supernatants (total volumes of supernatants were kept the same for each group) were collected for UV-vis-NIR (Varian Cary 5000, USA) analyses. Meanwhile, the intracellular SWCNTs were quantified by using SDS-PAGE method, as described previously.^[57] In detail, the cells were lysed with 160 μL lysis buffer (1% SDS, 1×10^{-3} M MgCl₂ and 1×10^{-3} M CaCl₂) for 3 min, then 20 μL cell lysates were subjected to SDS-PAGE electrophoresis on a standard BD Mini Vertical Gel (10 cm $\times$ 8 cm) with only 4% stacking gel as SWCNTs loading substrate. The samples were electrophoresed at 120 V for 2 h and SWCNTs would deposit on loading wells forming a band. After that, loading wells were sealed with 4% stacking gel. Next, UMAX scanner was used to scan SWCNTs bands on the gel and the intensity of the bands was analyzed using Gelpro software (Media Cybernetics, USA). To ensure

the loaded samples were from the same amount of cells, the protein amount of each sample was quantified with bicinchoninic acid protein assay kit (KangWei Co. Ltd., China).

Western-Blot Assay: After treatment, cell lysates (for detection of P2X₇R, PKC, ERK1/2, P38, and stress-activated protein kinase/JNK phosphoprotein) and culture supernatant (for cathepsin D (CTSD) protein detection) were separated by gradient separation gel (4%–20%) and 10% separation gel, respectively. The samples were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Germany) and the membranes were blocked with 5% nonfat milk and washed three times with tris buffered saline tween 20 (TBST) (containing 0.1% Tween 20). Thereafter, the membranes were incubated with primary antibodies (1:500–1:1000 dilution) against P2X₇R, phospho-PKC, phospho-p44/42, phospho-p38, phospho-JNK, β -actin, and cathepsin D (Cell Signaling Technology, MA, USA) at 4 °C for overnight, and then washed with TBST again. The membranes were incubated with corresponding HRP-conjugated secondary antibody (1:2000–1:5000 dilutions) for 1 h at room temperature, followed by three washes with TBST. The target proteins were observed using an ECL kit (Merck, Germany) and detected on X-ray film. Quantitative analysis of the band intensities was performed using Gel-pro image software (Media Cybernetics, USA). For CTSD measurement, the cells were exposed to SWCNTs for 5 h and the exposure solution was replaced with fresh medium, next, the supernatants (same volume supernatant for each group) were collected at indicated times for Western blot or UV-vis-NIR analyses.

Lysosensor Probe Labeling and Fluorescence Measurement: To monitor the pH change of lysosomes of cells upon SWCNTs exposure, two different lysosensor probes were used to label the cells separately so that to rule out the possibility that SWCNT may interfere with the fluorescence detection. In detail, Raw264.7 cells were seeded in 8-well chamber slide (Thermo) at the density of 1×10^5 cells per well in cRPMI and allowed to attach for overnight. The next day, the medium was replaced with 500 μL of cRPMI containing 2×10^{-6} M lysosensor DND-189 or 2×10^{-6} M lysosensor DND-153. After 30 min of incubation at 37 °C, the excess probes were removed by three washes with PBS. The cells were further treated with SWCNTs (1 and $2 \mu\text{g mL}^{-1}$) and 1×10^{-6} M ATP (positive control) for 2 h. After wash, the fluorescence images were acquired by using a confocal laser scanning microscopy (Leica, Mannheim, Germany) with excitation wavelength at 488 nm and emission wavelength at 505 nm. The fluorescence intensity was quantified by using LAS AF quantification software (Leica, Mannheim, Germany). The fluorescence intensity based on cell number was quantified and then the average fluorescence intensity of each cell was calculated.

Immunofluorescence Imaging for Microtubules Rearrangement: Raw264.7 cells were cultured at a density of 1×10^5 cells in an 8-well chamber slides (Thermo) and allowed to attach for overnight. The cells were treated with 2 and $5 \mu\text{g mL}^{-1}$ SWCNTs and 1×10^{-3} M ATP (positive control) for 5 h at 37 °C and then incubated for another 5 h in fresh media. Thereafter, the cells were fixed with 500 μL of formalin/PBS solution (4% formalin) for 15 min at room temperature. After washing with phosphate buffer saline tween 20 (PBST) (0.05% Tween20), the slides were blocked with 5% bovine serum albumin (BSA) in PBS for 1 h and incubated with anti- β -tubulin mouse monoclonal (1/200) antibody in PBS at

4 °C for overnight. After rinsing with PBST, the slides were incubated with 0.2 µg mL⁻¹ Hoechst 33342 for 30 min and washed and further cultured with Alexa Fluor 488 labeled goat anti-mouse IgG (1/200) for 2 h at room temperature in the dark. After washing with PBST, the slides were mounted with coverslips using Mowiol solution (Calbiochem, Merck, Germany). The immunostained cells were observed on an upright fluorescence microscope (ZEISS, Germany).

IL-1 β Secretion Measurement: Raw264.7 cells were treated with SWCNTs for 5 h, followed by replacing exposure solutions with fresh medium. The supernatant was collected at indicated times and centrifuged at 12 000 rpm for 20 min to remove particles and the concentration of IL-1 β in supernatant was measured via ELISA method as following: a 96-well plate (Nunc, Thermo) was coated with 200 µL anti-mouse IL-1 β mAb (1:250) and incubated at 4 °C for overnight. The next day, the plate was washed with PBS (pH = 7.0) containing 0.05% Tween 20 twice. Thereafter, the plate was incubated with 200 µL PBS containing 10% FBS at room temperature for 1 h, followed by washing with PBS. The supernatants were added to the plate and incubated for 2 h at room temperature. After washing, biotinylated anti mouse IL-1 β mAb (1:500) was added and incubated for 1 h at room temperature, followed by incubation with streptavidin-horseradish peroxidase conjugate for 30 min at room temperature. The 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent was added and the plate was incubated for 30 min at room temperature in the dark. The reaction was stopped by addition of 200 µL 2 M H₂SO₄. The absorbance at 450 nm was measured with a Varioskan Flash microplate reader (Thermo, MA, USA). In the experiment, the cells pretreated with ATP were set as positive control.

Cell Viability Measurement: Cells were seeded in a 96-well plate at a density of 2×10^4 cells per well and allowed to attach for overnight. The next day, cells were treated with ATP, oATP, or SWCNTs for indicated times. Cells were washed with PBS twice and incubated in cRPMI with 10% (w/w) WST-1 solution for 2 h. The absorbance at 450 nm of each well was measured with a Varioskan Flash microplate reader.

Statistical Analysis: All data analysis with two-tails student's t-test was completed in Microsoft office excel version 2013. Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. Significance asterisk * denotes * $p < 0.05$, ** $p < 0.01$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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