Published in final edited form as:
Nano Lett. 2005 September ; 5(9): 1676–1684. doi:10.1021/nl0507966.

Single-Walled Carbon Nanotube Induces Oxidative Stress and Activates Nuclear Transcription Factor-κB in Human Keratinocytes

Sunil K. Manna[†,‡], **Shubhashish Sarkar**[‡], **Johnny Barr**[‡], **Kimberly Wise**[‡], **Enrique V. Barrera**[§], **Olufisayo Jejelowo**[‡], **Allison C. Rice-Ficht**[||], and **Govindarajan T. Ramesh**[*,‡]
Molecular Neurotoxicology Laboratory, Department of Biology, Texas Southern University, Houston, Texas 77004, Deptartment of Mechanical Engineering and Materials Science, Rice University, Houston, Texas 77005, Immunology Laboratory, Center for DNA Fingerprinting & Diagnostics, Hyderabad AP-500076, India, and Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, College Station, Texas 77843

Abstract

Carbon nanotubes are now becoming an important material for use in day to day life because of their unique physical properties. The toxicological impact of these materials has not yet been studied in detail, thereby limiting their use. In the present study, the toxicity of single-walled carbon nanotubes (SWCNT) was assessed in human keratinocyte cells. The results show increased oxidative stress and inhibition of cell proliferation in response to treatment of keratinocytes with SWCNT particles. In addition, the signaling mechanism in keratinocytes upon exposure to SWCNT particles was investigated. Results from the study suggest that SWCNT particles activate NF-κB in a dose-dependent manner in human keratinocytes. Further, the mechanism of activation of NF-κB was due to the activation of stress-related kinases by SWCNT particles in keratinocytes. In conclusion, these studies show the mechanism of toxicity induced by SWCNT particles.

Introduction

Carbon nanotubes are tubular carbon molecules with properties that make them potentially useful in extremely small scale electronic and mechanical applications. They exhibit unusual strength and unique electrical properties and are extremely efficient conductors of heat.[1] Studies reported on carbon nanotubes to date have suggested their utility in a large number of sectors from microdevices and electronics to biological applications. The use of carbon nanotubes in various biological applications raises questions regarding the safety of its use and urges vigorous toxicological evaluations in various models.

Graphite and carbon fibers have had limited toxicological evaluations, but epidemiological studies have shown their potential toxic effect on the lungs. Studies have indicated an increased incidence of pneumoconiosis upon exposure to graphite-containing dust.[2,3] In more advanced studies, exposure to carbon nanotubes in rats has shown pulmonary injury resulting in multifocal granulomas.[4] In a recent study, exposure of human keratinocyte cells to carbon

*Corresponding author: Dr. G. T. Ramesh, Assistant Professor, Department of Biology, Texas Southern University, 3100 Cleburne St, Houston, TX 77004. Tel: 713 313-7784. Fax: 713 313-7932. ramesh_gt@tsu.edu.
[†]Center for DNA Fingerprinting & Diagnostics.
[‡]Texas Southern University.
[§]Rice University.
[||]Texas A&M University System Health Science Center.

nanotubes showed increased oxidative stress and accumulation of peroxidative products, followed by antioxidant depletion. The biochemical events described in this cell line resulted in a loss of cell viability and morphological changes.[5] In addition, exposure of a macrophage cell line to fullerenes or nano-structure (C_{60} and C_{60-70}) resulted in an increase in tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-8 (IL-8) in the culture supernatant. In conclusion, this work showed an increase in the inflammatory response upon exposure to carbon nanostructure particles.[6] In a preliminary study, Huczko et al.[7] tested fullerene soot containing C_{60} or carbon nanotubes for skin irritation and allergy risks. The results of dermatological trials did not show any signs of health hazard related to skin irritation and allergic risks.[7] However, it is suggeted that due to the unique properties of carbon nanaparticles it may lead to unique health hazards.[9]

Carbon nanoparticles are the material of the future, so evaluation of exposure to these materials requires toxicological evaluation in order to establish minimal standards to avoid health calamities in the future. Taken together, the evidence from carbon nanotube toxicity studies indicates the necessity to systematically define the basic mechanism(s) underlying their toxicity.

Carbon nanostructure is reported to induce TNF-alpha in a macrophage cell line and oxidative stress in keratinocytes.[5,6] In addition it is known that TNF-alpha and oxidative stress both causes induction of Nuclear Factor-kappa B (NF-κB), a known transcription factor.[9] The active NF-κB complex consists of three major proteins comprising P50, P65, and I kappa B kinases (IκB kinases). The activation of NF-κB is dependent on IκB kinases which phosphorylate IkappaB-alpha leading to its degradation and thereby releasing the active NF-κB (p50/P65 dimer) which is translocated to the nucleus.[9,10] The activated NF-κB then can bind to responsive elements within their promoters and transcribe genes. In addition, it has been suggested that the activation of NF-κB is regulated by some upstream mitogen-activated protein inases (MAPK) that regulate N treminal C-Jun Kinase (JNK) activation in the cells.[11]

From previous observations it seems likely that carbon nanoparticles can interfere with the NF-κB pathway because of their ability to induce TNF-alpha and oxidative stress.[5,6] The work described in this paper consists of an evaluation of the effect of carbon nanotubes in HaCaT cells to determine growth-inhibiting potential and oxidative stress. For the first time, we show the involvement of NF-κB in single-walled carbon nanotubes (SWCNTs) induced toxicity in HaCaT cells.

Results

SWCNTs were provided by Dr. Enrique Barrera**,** Department of Mechanical Engineering and Materials Science, Rice University, Houston, TX. In all the studies done here, SWCNT particles were dissolved in dimethylformamide (DMF) and therefore, in all control experiments, cells were treated with an equivalent volume of DMF. The cell lines used in this study were as follows: HaCaT (human keratinocytes), HeLa (epithelial cells), and A549 and H1299 (both human lung carcinoma) cells obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL).

SWCNT Particles Induce Oxidative Stress and Inhibit Cell Proliferation in HaCaT Cells

Induction of oxidative stress by SWCNT particles in HaCaT cells has been reported, and as a consequence significant cell death has been observed.[5] We assayed for similar effects in HaCaT cells to establish the system. To study the induction of oxidative stress induced by SWCNT

particles, HaCaT cells were exposed to different concentrations of SWCNT particles and generation of reactive oxygen species (ROS) was monitored through increases in fluorescence intensity of dichlorofluorescin (DCF). In brief, the ROS assay was performed as a modification of the method described earlier by Wise et al.[12] Intracellular accumulation of ROS was determined with 2′,7′-dichlorofluorescin diacetate (H_2DCF). H_2DCF is a nonfluorescent compound that accumulates within cells upon deacetylation and then reacts with ROS to form the fluorescent dichlorofluorescein (DCF). The cells were treated with different concentrations of SWCNT for 12 h, and increase in fluorescence was observed in the presence of H_2DCF (Molecular Probes, Eugene, OR, Figure 1). The increase in fluorescence was monitored in a fluorometer, and the values were normalized by protein content. The results show a dose-dependent increase in fluorescence compared to control cells thereby indicating generation of ROS by SWCNT particles in HaCaT cells. A significant increase in ROS is shown at concentrations ranging from 1 to 10 μg/ mL. This oxidative assault to cells could lead to a decrease in cell proliferation or even leading to cell death via an apoptotic pathway or by necrosis.[13–15]

To further assess the extent of damage as a result of oxidative assault by SWCNT particles, cell viability was determined after exposing the cells to various concentrations of SWCNT particles. Cytotoxicity was assayed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) dye uptake as described by Manna et al.[16] Briefly, HaCaT cells (10^4 cells/well of 96-well plate) were incubated with SWCNT particles in a final volume of 0.1 mL for 72 h at 37 °C. After the incubation 25 μL (5 mg/mL) of dye was added and incubated for 2 h before making a cell lysate to measure the absorbance at 570 nm. The results from this assay revealed a dose-dependent decrease in cell viability at concentrations as low as 0.5 μg/mL. This decrease was dose dependent with increasing concentrations of SWCNT particles (Figure 2A).

To reconfirm the cell viability results, a live-dead cell assay was performed essentially as described by Manna et al.[16] Briefly, following treatment with 10 μg/mL of SWCNT particles, approximately 10^5 cells were stained with Live/ Dead reagent (5 μM ethidium homodimer, 5 μM calcein-AM, Molecular Probes, Eugene, OR) and then incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). At a concentration of 10 μg/mL of SWCNT particles, there was a significant increase in dead cells as compared to the control and the increase was related as a function of time to the treatment (Figure 2b). The percentage of dead cells on treatment with SWCNT particles progressively increased from 31% to 68% across a time span of 72 h (Figure 2B) with no obvious change in cells treated with vehicle alone. These results suggest that exposure of HaCaT cells to SWCNT particles results in cell death which might be due to the excess of oxidative stress generated within the cells.

To study the specificity of induction of ROS by SWCNT particles, a similar assay was used to test three other human cell lines treated with various concentrations of SWCNT particles for 72 h. At the end of the 72 h incubation, cell viability was determined by MTT assay as described earlier.[16] HeLa, H1299, and A549 cells showed a similar loss of cell viability as was observed in HaCaT cells (Figure 3). HeLa cells were derived from human cervical cancer; H1299 and A549 cells are lung-derived. The results showed similar cell viability curves which suggests that a common mechanism might be associated with this phenomenon. Taken together, the results indicate that exposure of SWCNT particles to cells causes significant cell death at concentrations as low as 0.5 μg/mL. Cell death is probably due to the generation of oxidative stress coupled to a specific signaling mechanism.

Results from earlier studies have indicated that most downstream events observed were lipid peroxidation, loss of antioxidants, and accumulation of lipid peroxidative products upon

generation of ROS by treatment of SWCNT particles to HaCaT cells. Studies have also indicated loss of cellular morphology as a result of oxidative stress induced by SWCNT particles.[5] We therefore resorted to understand the downstream signaling events that could have resulted from the increase of ROS generated by SWCNT particles in HaCaT cells. To better understand the mechanism of its toxicity of SWCNT particles in HaCaT cells, it will require an understanding of the downstream signaling events that could have resulted from the increases of ROS generated by the SWCNT particles.

Activation of NF-κB by SWCNT Particles in HaCaT Cells

This group and others have previously shown that oxidative stress can activate NF-*κ*B, stress-activated kinases, and such activation could result in cell death by either apoptosis or necrosis.[17–19] In the next series of experiments the activation of NF-*κ*B was investigated by exposing HaCaT cells to various concentrations of SWCNT particles for 12 h and performing enzyme mobility shift assays (EMSA). EMSA was conducted essentially as described by Ramesh et al.[20] Briefly, 8 *μ*g of nuclear extract proteins was incubated with ^{32}P (Amersham, Piscataway, NJ) end-labeled 45-mer double-stranded NF-*κ*B oligonucleotide of HIV-LTR, 5′-TTG TTA CAA **GGG ACT TT**C CGC TG**G GGA CTT T**CC AGG GAG GCG TGG-3′ (boldface indicates NF-*κ*B binding site) for 30 min at 37 °C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The specificity of binding was examined by competition with unlabeled oligonucleotide. Visualization of radioactive bands was carried out by a PhosphorImager (Molecular Dynamics, BioRad, Hercules, CA) using ImageQuant software. Results in Figure 4A clearly show the activation of NF-*κ*B at concentrations as low as 1 *μ*g/mL SWCNT particles. The increase in NF-*κ*B protein complex binding to its cognate sequence was a dose-dependent event. These observations indicate that SWCNT particles could activate NF-*κ*B as a result of increased oxidative stress.[17–19]

As mentioned earlier, a dose-dependent increase in oxidative stress by SWCNT was observed and this increase is correlated with the activation of the NF-*κ*B in HaCaT cells (Figures 1 and 4). For the first time, there are data to suggest that SWCNT particles are capable of inducing NF-*κ*B in a dose-dependent manner in HaCaT cells. To further examine the binding specificity, EMSA was carried out in the presence of antibodies to p50 and p65 proteins (Santacruz Biotechnology, Santa Cruz, CA). p50 and p65 proteins translocate to the nucleus and bind to the DNA forming the active component of the NF-*κ*B complex.[9,10]

In Figure 4B supershift assays were carried out using antibodies specific to p50, to p65, and to nonspecific proteins. A supershifted band was observed in lanes 4, 5, and 6, confirming the protein complex bound was p50/p65 and which is the active complex required for DNA recognition followed by gene transcription. Under similar assay conditions, nonspecific antibodies added to the reaction mix failed to cause a bandshift: Anti-cyclin D1 (lane 7), Anti-c-Rel (lane 8), and pre-immune serum (lane 9). (All antibodies were purchased from Santacruz Biotechnology.) There was no binding in the nuclear extract from untreated cells (lane 2) or in the presence of a mutant oligo HIV-LTR 5′-TTG TTA CAA ***CTC*** ACT TTC CGC TGG GGA CTT TCC AGG GAG GCG TGG-3′, (boldface and italic bases represent the mutated site, lane 11). In presence of excess unlabeled oligos, the specific band was completely competed out indicating thereby the specificity of the assay (lane 10). These observations indicate that HaCaT cells exposed to SWCNT particles activate NF-*κ*B and strongly suggest a role for NF-*κ*B in the process of cytotoxicity.

NF-*κ*B is an important transcription factor and has been shown to participate in cell death and in inflammatory responses.[21] The hypothesis is that NF-*κ*B activation by SWCNT particles could lead to the binding of the activated complex to the promoter sequences and thus aid in

transcription. To test this hypothesis, a promoter assay was performed using secretory alkaline phosphatase (SEAP) as reporter gene. SWCNT-induced NF-κB-dependent reporter gene transcription was measured as previously described.[22,23] Briefly, HaCaT cells were transiently transfected by the calcium phosphate method with 1 mL of medium containing 0.5 μg of NF-κB promoter DNA linked to the heat-stable SEAP gene. The total amount of DNA was maintained at 3 μg by the addition of the control plasmid pCMVFLAG1 DNA. After 6 h of transfection, cells were washed, cultured for 12 h, and then treated with SWCNT particles for 24 h. Cell culture-conditioned medium was harvested, and 25 μL was analyzed for alkaline phosphatase activity essentially as per the CLONTECH protocol (Palo Alto, CA). As expected, there was a dose-dependent increase in reporter gene expression in the presence of various concentrations of SWCNT particles (Figure 5). These results indicate that SWCNT particles not only activate the NF-κB transcription factor but also allow this protein complex to bind the DNA and initiate transcription.

The activation of NF-κB is dependent on degradation of IκBα which is bound to p50/p65 and thus forming an inactive trimeric complex. To validate the specificity of the assay and the role of IκBα, HaCaT cells were transfected in parallel with overexpressing mutant IκBα construct into cells that lacks either Ser^{32} or Ser^{36} phosphorylation sites.[24] Therefore in the presence of mutant IκBα, phosphorylation of the endogenous IκBα is blocked and its degradation by ubiquitination is inhibited resulting in forming of the NF-κB complex. The translocation of active NF-κB (p50/p65) complex to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα.[21,25] However, in the presence of mutant IκBα this process is inhibited, and therefore the formation of active NF-κB complex is abrogated.[21] As expected, transfection of the mutant IκBα (refers to IκBα-dominant negative; IκBα-DN) construct in HaCaT cells did not show any SEAP activity in the presence of SWCNT particles. The reporter gene assay was very specific because no SEAP activity could be observed when dominant negative IκBα-DN was transfected into the same cells. As mentioned earlier, IκBα degradation is required to allow translocation of NF-κB to the nucleus which was confirmed by the failure to observe any SEAP activity in the presence of dominant negative form of the protein.[21,25] Since the IκBα-DN transfection did not show any promoter activity in the presence of SWCNT, it can be concluded that IκBα is phosphorylated followed by ubiquitination, thus allowing NF-κB to translocate to the nucleus where it activates gene transcription.

SWCNT Particles Induce Degradation of IκBα through Activation of IKK in HaCaT Cells

The degradation of IκBα in HaCaT cells after treatment with SWCNT particles for different periods of time was also examined using western analysis carried out according to the procedure previously described by Ramesh et al.[26] To correlate translocation of NF-κB into the nucleus preceded by degradation of IκBα, a gel shift assay of the nuclear extract was performed. The same extract was used for a western blot to identify IκBα in the cell lysate. An EMSA assay was performed in which there was clear binding of NF-κB in the extract as early as 2 h after treatment with SWCNT. This DNA binding was time-dependent when cells were treated with SWCNT at a concentration of 10 μg/mL (Figure 6A). Cytoplasmic extract from the same cells was subjected to western analysis to measure the degradation of IκBα as function of time. The results indicate that after 6 h of treatment with SWCNT particles, degradation of IκBα was observed. This shows that activation of NF-κB was followed by degradation of IκBα (Figure 6B). The degradation of IκBα was time-dependent and was observed for as long as 24 h after treatment with SWCNT particles. In the same extract, western blot was performed with β-actin antibody to ensure equal protein loading (Figure 6B). Blots were then analyzed by “Quantity One” software from BioRad Laboratories (BioRad, Hercules, CA), and involved calculation of the ratio between band intensities of IκBα and β-actin. Results from this analysis showed a significant decrease in IκBα protein after 6 h of treatment with SWCNT particles. The ratio

between band intensity of IκBα and band intensity of β-actin was then calculated for each time point using Quantity One′ software. The ratio values between IκBα and β-actin were for 3.1 ± 0.51 at 0 h, 2.75 ± 0.42 at 1 h, 3.4 ± 0.38 at 2 h, 0.56 ± 0.04 at 6 h, 0.68 ± 0.07 at 12 h, an 0.45 ± 0.04 at 24 h. There was perfect correlation between the activation of NF-κB and the degradation of IκBα. The analysis showed a decease in the ratio of the band intensities between IκBα and β-actin after 6 h of treatment with SWCT particles in HaCaT cells. The degradation of IκBα co-related with the activation of NF-κB and also suggests that SWCNT particles are capable of inducing the phosphorylation of IκBα and thus allowing the protein to degrade through the process of ubiquitination.

The sequential events of degradation of IκBα, followed by activation of NF-κB, is a classical pathway through which SWCNT particles act in the activation of NF-κB in HaCaT cells. This type of activation pathway has been reported for NF-κB.[21,25] The results obtained show that SWCNT particles can interfere with the endogenous signaling pathway for activation of NF-κB. The phosphorylation of IκBα occurs through the activation of IκB kinase (IKK). Since phosphorylation of IκBα was observed in HaCaT cells treated with SWCNT particles, the increase in activity of IKK may be one of the targets and is an upstream protein in the signaling cascade.

To get an insight as to the effect of SWCNT particles on NF-κB activation, an IKK activation assay was performed as described previously by Manna et al. 1998.[28] Briefly, IKK complex from whole-cell extract (300 μg) was precipitated with anti-IKKα antibodies (1 μg each), followed by treatment with protein A/G-Sepharose beads (Pierce, Rockford, IL). After a 2 h incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 20 μCi of (γ-^{32}P)-ATP, 10 mM unlabeled ATP, and 2 μg of GST-IκBα (aa1-aa54) as substrate. After incubation at 30 °C for 30 min, the reaction was terminated by boiling with SDS-PAGE sample buffer for 5 min. Finally, the protein was resolved on 9% SDS-PAGE, the gel was stained with Coomassie blue dye and dried, and the radioactive bands were visualized by PhosphorImager. As shown in Figure 6C, the radiolabeled glutathione-S-transferase-IκBα (GST-IκBα) band increased in intensity upon treatment with SWCNT particles at 2, 6, and 12 h. This suggests that SWCNT particles induce IKK activation that phosphorylates IκBα, which correlates with the phosphorylation and degradation of IκBα. Therefore, the activation pathway for the NF-κB by SWCNT particles is sequentially through the activation of IKK. IKK degrades IκBα through phosphoryltion, thereby allowing NF-κB complex to translocate into the nucleus for active transcription. Such pathways have been generally observed for cytokine-mediated activation of NF-κB and it would not be too speculative to state that SWCNT particles interfere with the cytokine pathways.[21,25] For example, IL-8 mediated activation of NF-κB has been shown to show a similar kind of activation pathway as described in the present communication.[28]

The active units of NF-κB complex essentially are p50/ p65 proteins, and they act as a transcription factor.[11] Both proteins require translocation from the cytosol to the nucleus, which occurs after the phosphorylated IκBα is degraded. To study the translocation of p65 protein into the nucleus, western analysis of the nuclear fraction and the cytosolic extract was performed. As evident in Figure 7A, the p65 protein started disappearing from the cytosol 2 h after treatment with SWCNT particles and translocated into the nucleus. The disappearance of p65 protein from the cytosol was not seen until the end of 24 h in HaCaT cells treated with SWCNT particles (Figure 7A). Western blot analysis for β-actin was performed to ensure equal loading of protein.

The ratio was then calculated for each pair of p65 and β-actin band intensities at various time points, using Quantity one software from BioRad. The ratio of band intensities of p65/band

intensity of actin protein for each pair for cytosolic fraction were at 1.2 ± 0.08 at 0 h, 1.4 ± 0.12 at 0.5 h, 1.6 ± 0.11 at 1 h, 0.6 ± 0.04 at 2 h, 0.42 ± 0.023 at 6 h, 0.38 ± 0.06 at 12 h, and 0.35 ± 0.05 at 24 h. The p65 protein was significantly depleted in the cytosol after 2 h of incubation with 10 μg/mL of SWCNT for 12 h in HaCaT cells. In addition, the appearance of the p65 band in the nuclear extracts of the same cells was observed after 2 h of treatment and increased with time until the end of 24 h of treatment with SWCNT particles (Figure 7B). Under similar conditions to western blot for CRM1, a nuclear protein, showed no change in band intensity, verifying equal protein loading across all the wells in the blot and the authenticity of the nuclear extract preparation. The band intensity ratio of p65 to band intensity of CMRI protein was calculated for the nuclear fraction at various time points. The ratios of the intensities were 0.02 ± 0.005 at 0 h, 0.017 ± 0.003 at 0.5 h, 0.022 ± 0.004 at 1 h, 0.025 ± 0.06 at 2 h, 0.56 ± 0.03 at 6 h, 0.8 ± 0.02 at 12 h, and 0.88 ± 0.06 at 24 h. The intensity ratio increased as a function of time of exposure to SWCNT particles to HaCaT cells. The results show that treatment of SWCNT particles to HaCaT cells induces translocation of p65 protein into the nucleus, supporting the activation of NFκB. p50 protein which is one of the other active protein in the NFκB complex should have also translocated into the nucleus. This is evident by the fact that treatment of SWCNT paticle showed increased NFκB promoter activity in our reporter gene assay experiment and such observation is possible only when p50/p65 protein dimer translocates into the nucleus (Figure 5). All these observations taken together show that treatment of HaCaT cells with SWCNT particles activate NF-κB through the activation of p50 and p65 proteins.

SWCNT Particles Induce Mitogen-Activated Protein Kinase (MAPK) Activation

Several lines of evidence suggest that mitogen-activated protein kinases (MAPK) and c-Jun M-terminal kinase (JNK) can participate in the regulation of NF-κB transcriptional activity.[12,29] Therefore, an investigation into the effect of SWCNT particles on the activation of MAPK was performed by western blot as described by Manna and Ramesh.[28] Twelve hours of treatment with various concentrations of SWCNT particles caused a significant increase in tyrosine phosphorylation of p42 and p44 MAPK as detected by western blot using specific antibody reacting to tyrosine phosphorylated p42 and p44 MAPK. The increase in the phosphorylation was evident at a doses 1, 5, and 10 μg/mL. However, the extracts from the same cells did not show any effect on total MAPK as detected in the western blot using specific antibody at any of the doses of SWCNT particles tested in parallel for phosphorylation. The results show that HaCaT cells treated with SWCNT particles induced phosphorylation of MAPK in a concentration-dependent manner (Figure 8). The same blots were probed with anti-β-actin antibody as a marker for equal loading of protein. It is concluded that SWCNT particles can activate MAPK by inducing phosphorylation and also that this phosphorylated protein then can transactivate NFκB in the downstream of the signaling cascade. It is therefore argued that downstream signaling events triggered by SWCNT particles in HaCaT cells can be attributed to NF-κB activation possibly through the MAPK activation pathway. A similar activation of NF-κB through the involvement of MAPK has been described earlier for interleukin 8 (IL8) in U937 cells.[28] It is likely that SWCNT particles may be interfering with signaling events involved in inflammatory responses. Further, the activation of NF-κB could lead to induction of many other genes regulated by this transcription factor.[11]

Summary and Conclusions

The present study was undertaken to understand the downstream mechanisms involved in SWCNT toxicity. It has already been shown that SWCNT toxicity induces ROS in human keratinocyte cells.[5] In HaCaT cells, Shvedova et al. have previously shown ROS generation by SWCNT particles. The same effect has been observed here through similar experimental

stategy which thereby strengthens the hypothesis that SWCNT particles can induce ROS which may be responsible for the cytotoxicity (Figure 1). In addition use of different concentrations of SWCNT particles and variation of time periods for cell treatment has confirmed these observations. The results in this communication indicate that inhibition of cell growth by SWCNT particles may be a common cytotoxic response because HaCaT cells, and cells of different origin, HeLa, H1299, and A 549 cells, all showed similar loss of cell viability (Figure 3). Therefore cytotoxicity by SWCNT particles was seen as a common effect in the cell lines tested in this study. These results reveal that there might be a specific signaling mechanism being triggered by SWCNT particles and that the down stream effect of this pathway leads to cell death.

Studies to identify the specific signaling pathway indicate that one of the downstream events that is triggered upon treatment with SWCNT particles may be the activation of the NF-κB. Previous studies have shown increased levels of tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-8 (IL-8) in the supernatant of macrophage cell cultures exposed to fullerenes (C_{60} and $C_{60\text{-}70}$) when compared to control macrophage cultures.[6] Other studies have claimed no measurable induction of inflammation in respiratory tracts of guinea pigs exposed to SWCNT particles.[7] However, in rats exposed to carbon nanotubes there was formation of multifocal granulomas as evident by foreign tissue body reaction. Similar evidence indicate that SWCNT particles are capable of interfering with the cytokine signaling, resulting in inflammation, release of TNFα, IL6, and IL8.[6] Further several studies have shown the involvement of NFκB in inflamatory responses so activation of NFκB by SWCNT particles in HaCaT cells is reasonable to attribute to the cytotoxic effect of these particles.

Consistent with other studies, this work also shows a reduction in cell proliferation and generation of ROS. In addition there is activation of NF-κB by SWCNT particles in keratinocytes (Figure 4). Since NF-κB is involved with cytokine-mediated signaling, it could be speculated that SWCNT particles can interfere with or mimic cytokine signaling which might be the cause of the inflammation observed by earlier investigators.[6] In the present study, it has been shown that SWCNT particles induced activation of NF-κB through a pathway already characterized for many of the cytokines.[21,28] NF-κB activation followed the signaling cascade probably through the activated MAPK kinase (Figure 8). In addition, it was observed that down stream events in this signaling pathway were through the IKK kinase, which is a typical NF-κB activation pathway (Figure 6).

This study also shows a detailed mechanism for SWCNT particle-induced toxicity in keratinocytes. In conclusion, SWCNT particles show an adverse effect on keratinocytes through an oxidative mechanism leading to NF-κB activation. These results indicate the need for future investigations into the toxicity of SWCNT particles in different cell lines and in animal models. It would be interesting to see whether treatment with antioxidants could rescue cells from the adverse effects of SWCNT particles. Future experiments are needed to see if it is possible to counteract the adverse effects of SWCNT toxicity by using antioxidants or use of specific inhibitors for blocking NF-κB activation.

Acknowledgments

This work was supported by NASA funding NCC-1-02038: NCC 9-165: NAG 9-1414: NIH/ RCMI RR03045-18 (GR).

References

1. Endo M, Hayashi T, Kim YA, Terrones M, Dresselhaus MS. Philos Trans R Soc London, Ser A 2004;362:2223–2238.
2. Baron PA, Maynard AD, Foley M. NIOSH Rep. 2002NIOSH DART-02-191

3. Joseph G. DuPont Co Internal Report. 2002
4. Lam C, James JT, McCluskey R, Hunter RL. Toxicologist 2003;72(S1):44.
5. Shvedova AA, Castranova V, Kisin ER, Murray AR, Schwegler-Berry D, Gandelsman VZ, Maynard A, Baron P. J Toxicol Environ Health 2003;66:1901–1918.
6. Adelmann, P.; Bairerl, T.; Drosselmeyer, E.; Politis, C.; Polzer, G.; Seidel, A.; Schwegler-Berry, D.; Steinleitner, C. Effects of fullerenes on alvelor macrophages in vitro. In: Mohr, U.; Dungworth, DL.; Mauderly, J.; Oberddoerster, G., editors. Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract. ILSI Press; Washington, DC: 1994. p. 405-407.
7. Huczko A, Lange H. Fullerene Sci Technol 2001;9:247–250.
8. Borm PJA. Particle toxicology: From coal mining to nantotechnology. Inhalation Toxicol 2002;14:311–324.
9. Baichwal VR, Baeuerle PA. Curr Biol 1997;7:94–96.
10. Baeuerle PA, Baiscwal VR. Adv Immonol 1997;65:111–137.
11. Aggarwal BB, Natarajan K. Eur Cytokine Network 1996;7:93–124.
12. Wise CK, Manna SK, Yamauchi K, Ramesh V, Wilson BL, Thomas RL, Sarkar S, Kulkarni DA, Pellis NR, Ramesh GT. In Vitro Cell Dev Biol: Anim. in press
13. Lee FS, Hagler J, Chen ZJ, Maniatis T. Cell 1997;88:213–222. [PubMed: 9008162]
14. Takahashi A, Masuda A, Sun M, Centonze VE, Herman B. Brain Res Bull 2004;62:497–504. [PubMed: 15036564]
15. Higuchi Y. Cell Mol Med 2004;8:455–464.
16. Manna P, Narang KK, Manna SK. Int J Cancer 2004;113:549–560. [PubMed: 15389516]
17. Felix M, Manna SK, Wise K, Barr J, Ramesh GT. J Biochem Mol Toxicol 2005;19:67–77. [PubMed: 15849723]
18. Zhou YM, Zhong CY, Kennedy IM, Leppert VJ, Pinkerton KE. Toxicol Appl Pharmacol 2003;190:157–169. [PubMed: 12878045]
19. Li X, Stark GR. Hematolology 2002;30:285–296.
20. Ramesh GT, Sunil KM, Aggarwal BB, Jadhav AL. Toxicol Lett 2001;123:195–207. [PubMed: 11641047]
21. Aggarwal BB. Cancer Cells 2004;6:203–208.
22. Sarkar A, Sreenivasan Y, Ramesh GT, Manna SK. J Biol Chem 2004;279:33768–33781. [PubMed: 15161907]
23. Manna SK, Mukhopadhyay A, Aggarwal BB. J Immunol 2000;164:6509–6519. [PubMed: 10843709]
24. Sreenivasan Y, Sarkar A, Manna SK. Oncogene 2003;22:4356–4369. [PubMed: 12853972]
25. Karin M, Ben-Neriah Y. Annu Rev Immunol 2000;18:621–663. [PubMed: 10837071]
26. Ramesh GT, Manna SK, Aggarwal BB, Jadhav AL. Toxicol Appl Pharmacol 1999;155:280–286. [PubMed: 10079214]
27. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. J Biol Chem 1998;273:13245–13254. [PubMed: 9582369]
28. Manna SK, Ramesh GT. J Biol Chem 2005;280:7010–7021. [PubMed: 15591054]
29. Karin M. J Biol Chem 1995;270:16483–16486. [PubMed: 7622446]

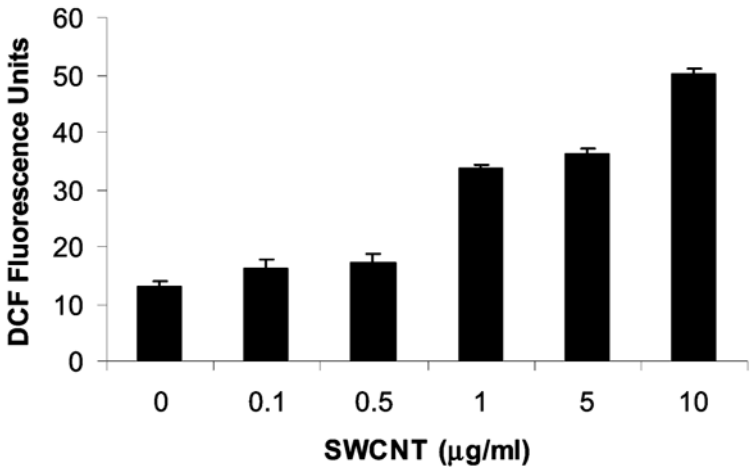

Figure 1.

Induction of reactive oxygen species (ROS) by SWCNT particles in HaCaT cells. HaCaT cells were treated with different concentrations of SWCNT particles for 12 h. Cell extracts were prepared and ROS generation was measured as described in the text. Results indicated are the mean (±standard deviation) fluorescence units from six independent experiments.

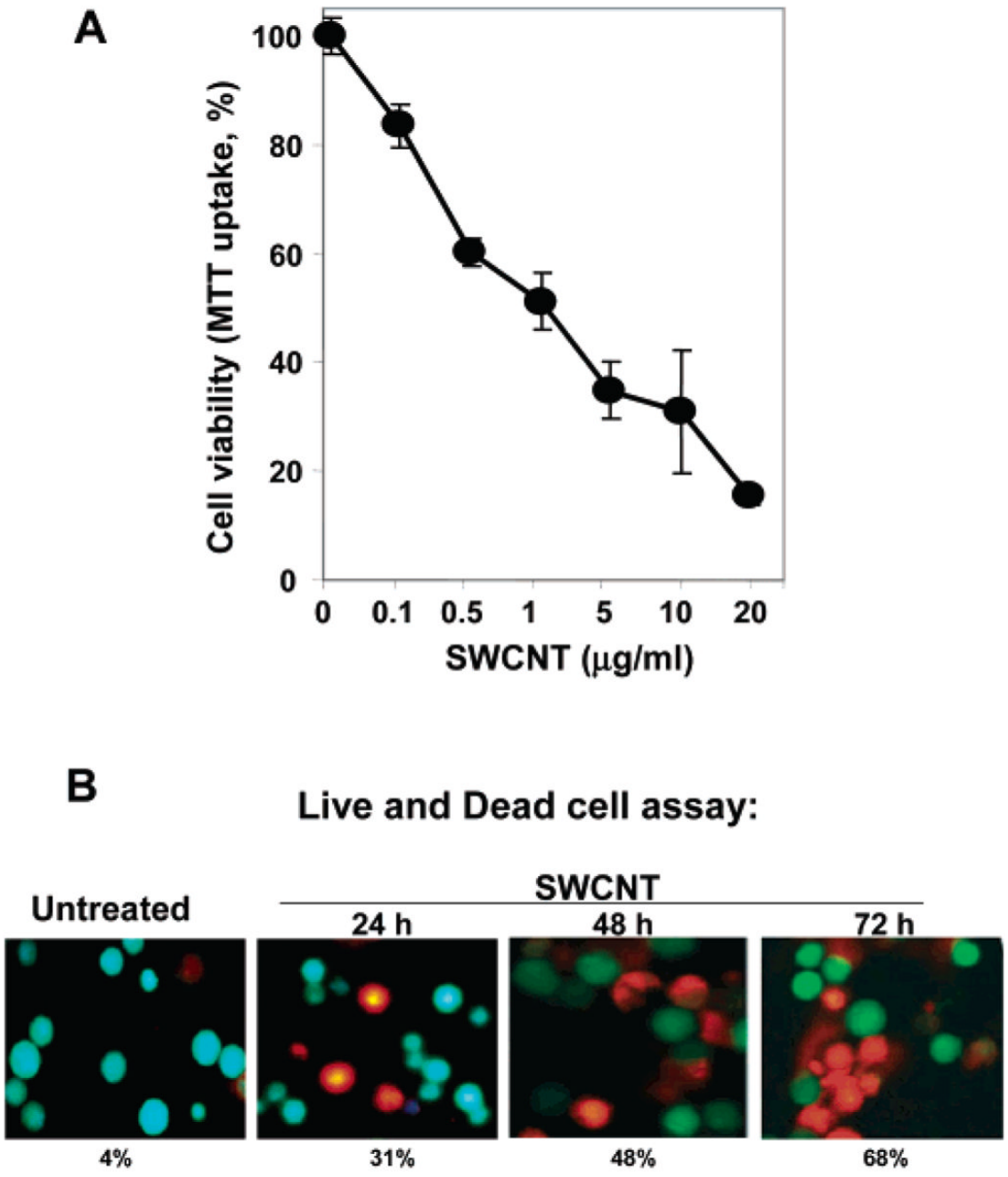

Figure 2.

Effect of SWCNT particles on cell viability. HaCaT cells (5000/well in a 96-well plate) were incubated for 12 h and then treated with different concentrations of carbon nanotubes for 72 h. Cell viability was assayed by MTT dye uptake (A). Results are reported as percentage of cell viability and representatives from one of three independent experiments. (B) HaCaT cells were treated with 10 μg/mL SWCNT for different time intervals, and the dead cell (red color) numbers were counted. The percentage of dead cells is indicated below each photograph.

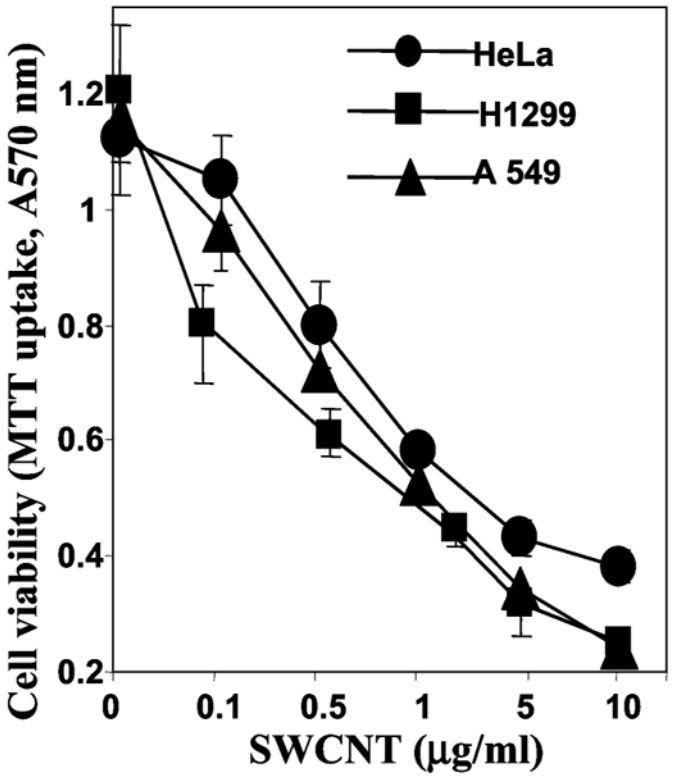

Figure 3.
Effect of SWCNT particles on cell viability in different cells. HeLa, H1299, and A549 cells (5000/well in a 96-well plate) were incubated with different concentrations of SWCNT for 72 h. Cell viability was determined by MTT assay.

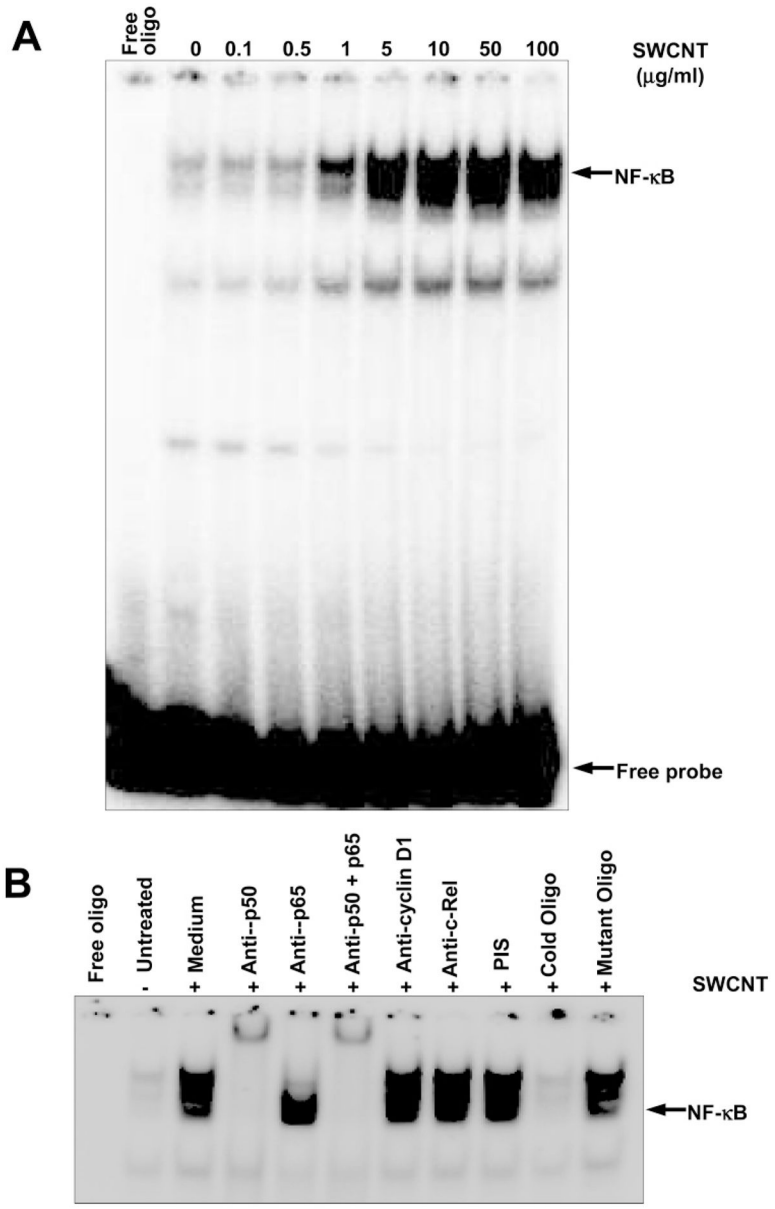

Figure 4.

(A) Effect of SWNCT particles on NF-*κ*B activation. HaCaT cells were treated with different concentrations of SWNCT for 12 h. Cells from these treatments were washed in chilled buffer to prepare cytoplasmic and nuclear extracts. Eight micrograms of nuclear extracts was used to assess the NF-*κ*B binding by electrophoretic mobility shift assay as described in the text. (B) Band supershift and specificity of NF-*κ*B activation. Nuclear extracts prepared from untreated cells or cells treated with SWNCT particles were incubated for 15 min with different antibodies, unlabeled oligo- and mutated NF-*κ*B oligonucleotides, before resolution of the binding complex in a 6.6% gel.

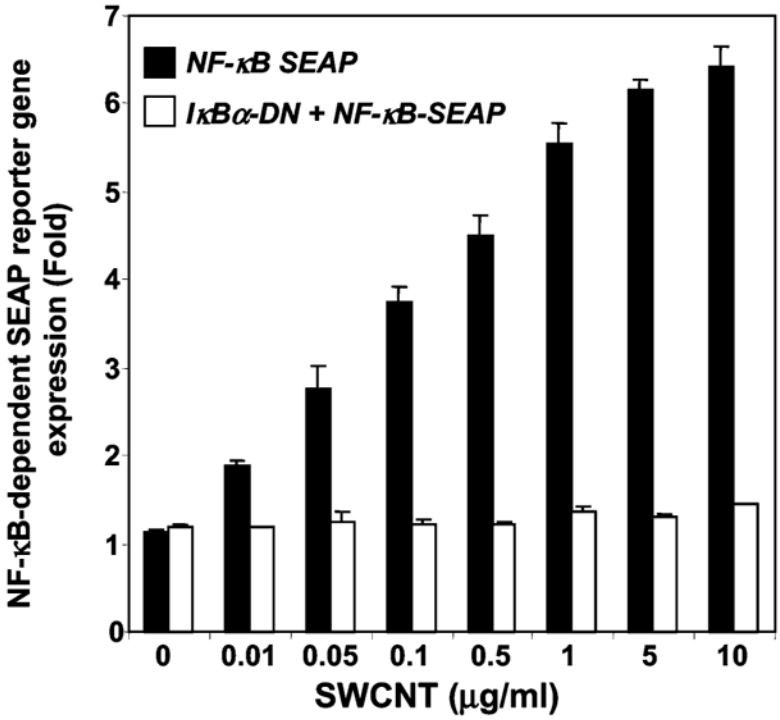

Figure 5.

HaCaT cells were transiently transfected with a NF-κB-containing plasmid linked to the SEAP gene with or without dominant negative IκBα plasmid for 6 h. Cells were then cultured for 12 h followed by stimulation with different concentrations of carbon nanotubes for 24 h. Culture supernatants were assayed for secreted alkaline phosphatase activity. Results are expressed as fold activity over the nontransfected control.

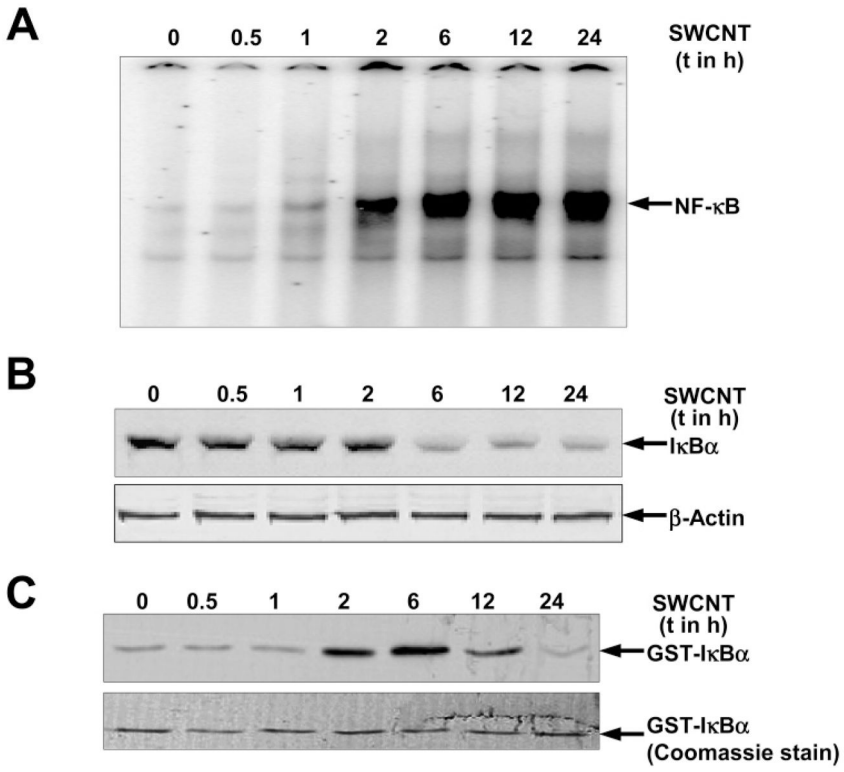

Figure 6.

Effect of SWNCT particles on NF-*κ*B activation and I*κ*Bα degradation. (A) HaCaT cells were treated with 10 *μ*g/mL SWNCT for different time intervals as indicated, before preparing cytoplasmic and nuclear extracts. Eight micrograms of nuclear extracts was assayed for NF-*κ*B by electrophoretic mobility assay. (B) Cytoplasmic extracts (50 *μ*g/sample) were assayed for I*κ*Bα by western blot. The same blot was reprobed with *β*-actin antibody to ensure equal protein loading. (C) Effect of carbon nanotubes on IKK activation. HaCaT cells were stimulated with 10 *μ*g/mL carbon nanotubes for different times as indicated and whole cell extracts were prepared. Two hundred fifty micrograms of protein was immunoprecipitated with anti-IKKα antibody and IKK activity was assayed using GST-I*κ*Bα as a substrate. The gel was stained with Coomassie brilliant blue and scanned. Radioactive bands were detected in a PhosphorImager.

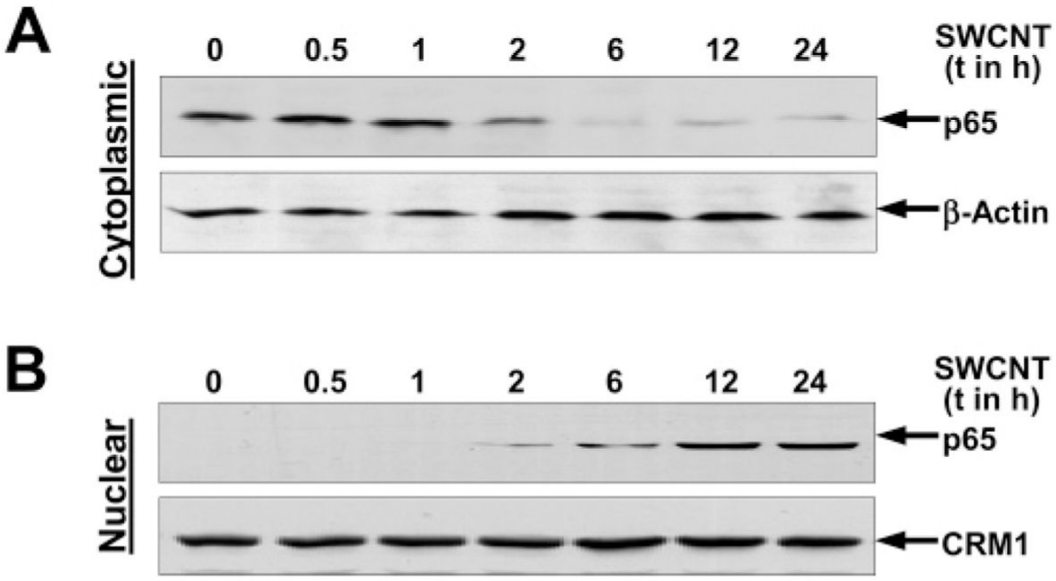

Figure 7.

Effect of SWNCT on translocation of p65 protein into the nucleus. HaCaT cells were treated with 10 μg/mL of SWNCT particles for 12 h and then 50 μg of cytoplasmic (A) and nuclear (B) extracts were resolved on 10% SDS-PAGE and assayed for p65 by western blot analysis. For equal protein loading of cytoplasmic extracts, the blot was stripped off and reprobed with anti-β-actin antibody and for nuclear extracts with anti-CRM1 antibody.

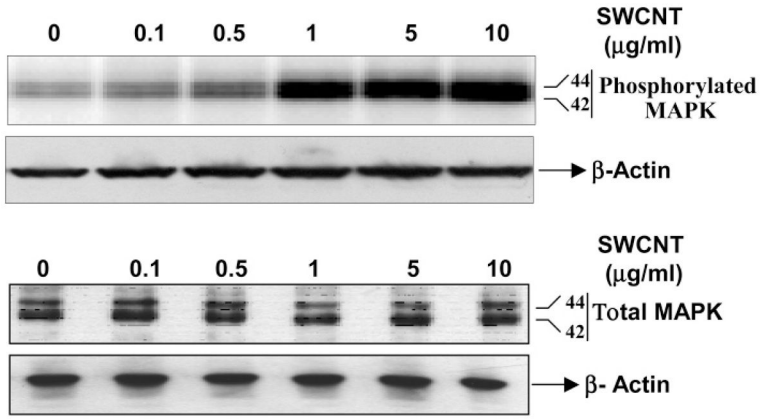

Figure 8.

Effect of SWNCT particles on MAPK activation. HaCaT cells were stimulated with different concentrations of SWNCT particles for 12 h at 37 °C. The cells were then washed and resolved on 10% SDS-PAGE and assayed for phosphorylated MAPK by western blot using anti-$P^{42/44}$ phosphorylated MAPK antibody and by antibody specific for total MAPK. The blots were stripped off and reprobed with anti-*β*-actin antibody to ensure equal loading of protein.