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Increased calcium influx in a monocytic cell line on exposure to ultrafine carbon black

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ABSTRACT: Ultrafine particles have been shown to induce pro-inflammatory effects both *in vivo* and *in vitro*. Increased expression of pro-inflammatory genes probably requires the activation of specific transcription factors such as nuclear factor kappa B (NF-κB) *via* a number of possible pathways including Ca^{2+} and reactive oxygen species.

The fluorescent dye fura 2, was used to measure cytosolic Ca^{2+} in the human monocytic cell line, Monomac 6 on exposure to 66 $\mu g \cdot mL^{-1}$ of either ultrafine carbon black (ufCB; diameter 14 nm), carbon black (CB; diameter 260 nm), quartz (diameter 1.45 μm), or medium alone.

UfCB but not fine CB induced a 1.6-fold increase ($p<0.01$) in the resting cytosolic Ca^{2+} concentration of Monomac 6 cells. In addition ufCB induced a 2.6-fold increase ($p<0.001$) in the response to the endoplasmic reticulum Ca^{2+}- adenosine triphosphatase (ATPase) inhibitor, thapsigargin, suggesting the Ca^{2+} release-activated Ca^{2+} current across the plasma membrane was enhanced. This response was inhibited by the removal of extracellular Ca^{2+} and by the Ca^{2+} channel blocker, verapamil. In addition, ufCB stimulated the entry of extracellular Mn^{2+}. Finally, the antioxidants mannitol and nacystelin both inhibited the effects of ufCB on the response to thapsigargin.

These data suggest that ultrafine carbon black particles stimulated an increase in cytosolic Ca^{2+}, possibly through the entry of extracellular Ca^{2+} *via* Ca^{2+} channels in the plasma membrane. The particles may in part activate the opening of Ca^{2+} channels *via* a mechanism involving reactive oxygen species.

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Ultrafine particles (particles with a diameter <~100 nm) for example, ultrafine titanium dioxide (25 nm diameter) [1] and ultrafine carbon black (ufCB) (14 nm diameter) [2] on exposure to the rat lung have been shown to be highly inflammogenic compared to larger particles of a similar chemical composition (250 and 260 nm, respectively). In addition, ultrafine particles have received considerable attention in relation to their potential role in the adverse health effects of environmental particulate air pollution (particles with a 50% cut-off aerodynamic diameter of 10 μm (PM_{10})) [3]. Several studies have also suggested that the transition metal content of PM_{10} and other particulates such as residual oil fly ash may be responsible for their biological reactivity [4, 5]. Much interest has focused on the pro-inflammatory effects of such particles in relation to the stimulation of pro-inflammatory cytokine production by target lung cells and macrophages [2, 5]. Candidate transcription factors for the activation of cytokine genes upon exposure to ultrafine particles include nuclear factor-κB (NF-κB) [6] and activator protein-1 (AP-1) [7, 8]. Several studies into the activation of such transcription factors by respirable particles have supported a role for oxidative stress [8, 9].

A large variety of stimuli have the ability to activate NF-κB including both Ca^{2+} [10] and reactive oxygen [11, 12]. Ca^{2+}, released from the endoplasmic reticulum (ER) by inositol 1,4,5-trisphosphate (IP_3) is known to activate a number of pro-inflammatory transcription factors such as NF-κB and nuclear factor of activated T-cells (NF-AT) [13]. Several types of pathogenic particles have been shown to induce alterations in cellular Ca^{2+} homeostasis which could underlie their pro-inflammatory effects [14, 15]. For example, Ca^{2+} activates the Ca^{2+}-dependent phosphatase, calcineurin, which activates pathways leading to dephosphorylation of cytosolic complexed transcription factors [16] and hence their activation. Hence Ca^{2+} is a suitable candidate for the signal between particle exposure and the activation of pro-inflammatory genes *via* transcription factors.

Based on the premise that the expression of pro-inflammatory genes can be switched on *via* intracellular Ca^{2+}, and that ultrafine particles induce an inflammation in the rat lung, the authors hypothesized that ultrafine particles, but not larger respirable particles, are able to alter intracellular Ca^{2+} signalling in macrophage cells.

The present study used a human monocytic cell line (Monomac 6; MM6) [17] loaded with a fluorescent dye (fura 2) sensitive to Ca^{2+} [18] before exposing to either carbon black (CB), or ultrafine carbon black (ufCB) [19] (table 1).

Table 1. – Physical characteristics of relevant particles

Particle	Mean diameter nm	Surface area $m^2 \cdot g^{-1}$	Iron content
CB	260.2±13.7	7.9	not detectable
ufCB	14.3±0.6	253.9	19 $ng \cdot mg^{-1}$
Quartz	800 (5000 maximum)	7.0	43 ng·mL*

*: unpublished data. CB: carbon black (Huber 990; Degussa, Germany); ufCB: ultrafine carbon black (Printex 90; Degussa).

The effects of particle exposure on the IP_3 sensitive ER Ca^{2+} store were estimated by the addition of the lactone, thapsigargin [20]. Thapsigargin acts by inhibiting the ER Ca^{2+}-adenosine triphosphatase (ATPase), the function of which is to sequester cytosolic Ca^{2+} into the ER. Exposure to thapsigargin leads to release of the ER Ca^{2+} store and hence an increase in cytosolic Ca^{2+} concentration which stimulates the entry of extracellular Ca^{2+} *via* Ca^{2+} release-activated Ca^{2+} (CRAC) channels [21] leading to a sustained increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). Manipulation of this technique allows investigation of the mechanism by which any alterations in Ca^{2+} homeostasis arise [14].

Materials and methods

Materials

The following materials were purchased from the sources stated: A549 cells (European Type culture collection), Roswell Park Memorial Institute (RPMI)-1640 medium, foetal calf serum (FCS), Hank′s balanced salt solution (HBSS), penicillin-streptomycin, L-glutamine, minimal essential medium (MEM) nonessential amino acids (Life Sciences International Ltd, Basingstoke, Hampshire UK), fine CB, ufCB (Degussa, Frankfurt, Germany), oxaloacetic acid, mannitol, trypan blue, holo-transferrin, verapamil, thapsigargin, fura 2-acetoxymethyl (fura2-AM), phosphate buffered saline (PBS), sodium pyruvate, dimethyl sulphoxide (DMSO) and $MnCl_2$ (Sigma-Aldrich Company Ltd, Poole, Dorset, UK). Nacystelin was kindly provided by SMB Pharmaceuticals (Brussels, Belgium). All other chemicals were of reagent grade.

Fura2-AM (1 $\mu g \cdot \mu L^{-1}$), thapsigargin (500 μM) and verapamil (10 mM) were all dissolved in DMSO and stored as frozen aliquots. Subsequent dilutions were all made in culture medium to minimize the introduction of DMSO into the experiment.

The fine CB (Huber 990; Degussa) and ufCB (Printex 90; Degussa) have been described in detail elsewhere [19, 22], but are summarized in table 1. The size of the particles was assessed by electron microscopy and the surface area by Brunauer, Emmett and Teller (BET) nitrogen adsorption.

Study design

MM6 cells were loaded with a fluorescent dye to measure intracellular Ca^{2+} ($[Ca^{2+}]_i$ before treating with particles. Cells were exposed to either medium alone or medium containing particles (66 $\mu g \cdot mL^{-1}$) for 2,000 s followed by stimulation with thapsigargin. The end-points measured included the slope of the $[Ca^{2+}]_c$ 200–2,000 s, the $[Ca^{2+}]_i$ at 2,000 s and the $[Ca^{2+}]_i$ after the addition of thapsigargin. Thapsigargin was added in order to assess whether the increase in cytosolic Ca^{2+} observed on treatment with particles was caused by depletion or leak of the ER Ca^{2+} store.

To investigate whether particles increased the influx of extracellular Ca^{2+} three strategies were employed. Either the Ca^{2+} channel blocker verapamil was added to the cells prior to the particles or, the cells were suspended in a low-Ca^{2+} or ethylene glycol tetra acetic acid (EGTA) containing medium before adding particles or, the cells were suspended in a Ca^{2+}-free medium which was supplemented with $MnCl_2$.

To investigate the role of reactive oxygen species, cells were treated with the antioxidants mannitol (2 mM) or nacystelin (400 μM) prior to the addition of particles.

Cell culture

The MM6 cells were obtained from the European collection of animal cell cultures [17]. These cells were cultured in a complex medium containing RPMI-1640 medium with 20% FCS, penicillin-streptomycin (30 $IU \cdot mL^{-1}$), L-glutamine, holo-transferrin (1 $\mu g \cdot mL^{-1}$), sodium pyruvate (1mM), MEM nonessential amino acids (1%) and oxaloacetic acid (1 mM) at 37°C, 5% CO_2.

MM6 cells ($4.5 \times 10^6 \cdot 10\ mL^{-1}$) were centrifuged ($900 \times g$, 2 min) and the pellet resuspended in 1 mL of PBS to remove the serum containing medium. Again the cells were centrifuged and the pellet resuspended in Hepes (23 mM)-buffered complex medium (0% FCS) containing fura2-AM (2 $\mu g \cdot mL^{-1}$ final concentration). The cell suspension was incubated in a shaking water bath at 34°C for 20 min to allow efficient loading of the membrane permeable fura2-AM, and cleavage of the acetoxymethyl (AM) group by intracellular esters to yield the Ca^{2+} sensitive, membrane impermeable dye, fura 2. After loading with fura2- AM the cells were centrifuged and the pellet resuspended in Hepes-buffered complex medium (20% FCS) to give 3×10^6 $cells \cdot mL^{-1}$.

Measurement of intracellular calcium

The cells were transferred to a quartz cuvette and the fluorescence measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm (LS-50B Luminescence Spectrometer; Perkin Elmer, Buckinghamshire, UK). During the fluorescence measurements the cells were maintained in suspension using a magnetic stirrer and the cuvette was thermostatically controlled at 37°C. The ratio of the fluorescence values at excitation wavelengths of 340 and 380 nm were calibrated and converted to Ca^{2+} concentration (nM) according to the protocol of GRYNKIEWICZ et al. [18] as follows.

$$[Ca^{2+}]_c = \frac{K_d(R - R_{min})}{(R_{max} - R)}$$

K_d is 224 nM, the apparent dissociation constant for Ca^{2+} and fura 2. The maximum ratio (R_{max}) was obtained by the addition of Triton X-100 (0.5%) to lyse the cells. The minimum ratio (R_{min}) was obtained by the addition of EGTA (7 mM, added as a 0.5 M stock buffered with 3M tris-hydroxymethyl-amino methase (Tris)-HCl).

Treatment of the MM6 cells with particles and reagents

All particles and reagents were added directly to the suspension of cells in the quartz cuvettes while fluorescence ratio measurements continued. Dusts were suspended (1 $mg \cdot mL^{-1}$) in medium and sonicated for 10 min to allow dispersion of the particles. Particles were added 400 s after the commencement of fluorescence ratio measurements. After exposure of the MM6 cells to the particles for the times specified, thapsigargin (dissolved in DMSO) was added to give a final concentration of 100 nM (DMSO did not exceed 0.5% final concentration).

Treatment of MM6 in low-calcium medium or EGTA

To investigate the responses of MM6 to particles in Ca^{2+}-free medium, the cells were suspended in a prewarmed (37°C) Ca^{2+}, magnesium-free HBSS with or without EGTA (400 µm final concentration) before transfer to the fluorimeter. Particles subsequently added were suspended in the Ca^{2+}, Mg^{2+}-free HBSS with or without EGTA. Low-Ca^{2+} experiments were also completed in the presence of $MnCl_2$ (100 µm final concentration) [23].

Data and statistical analysis

All experiments were carried out in triplicate, but on separate days, and the data are presented as mean±SEM unless otherwise stated. Statistical analysis was carried out using either a two sample t-test assuming equal variances, a one-way analysis of variance (ANOVA) according to similar publications [*e.g.* 24], or a Tukey's multiple comparison test using a level of significance "alpha" of 0.05 for "all" comparisons.

Results

Effect of ultrafine carbon black and carbon black on the release of calcium from the intracellular stores by thapsigargin

The resting $[Ca^{2+}]_c$ of the MM6 cell line in complete medium containing 20% FCS was 38.34±4.96 nM (n=24). Treatment of the MM6 cells with thapsigargin induced release of the ER Ca^{2+} store resulting in an increase in the $[Ca^{2+}]_c$ reaching 402.30±24.27 nM (n=12) (fig. 1).

MM6 cells were treated for 2,000 s (33 min) either with medium alone or with particles added to a final concentration of 66 $\mu g \cdot mL^{-1}$. During all treatments there was a steady increase in the resting $[Ca^{2+}]_c$ over time (fig. 1), probably due to the increase in temperature (from 34°C while loading with fura2-AM, to 37°C in the cuvette) and because of adjustment to the culture conditions (readdition of FCS to the medium). The increase in resting $[Ca^{2+}]_c$ was not due to a decrease in viability as assessed by trypan blue exclusion (data not shown) nor the response to thapsigargin (see below). Treatment of the MM6 cells with ufCB (66 $\mu g \cdot mL^{-1}$ final concentration) for 2,000 s resulted in a significant increase in the slope of the resting $[Ca^{2+}]_c$ to 0.12±0.02 $nM \cdot s^{-1}$ (n=5) compared to the control slope of 0.089±0.004 $nM \cdot s^{-1}$ (n=8; $p<0.05$). As a consequence of the increased slope at the end of the 2,000 s treatment with ufCB (20% FCS) the resting $[Ca^{2+}]_c$ was significantly higher (140.23±16.54 nM) compared to the untreated cells (86.84±14.00 nM; $p<0.01$).

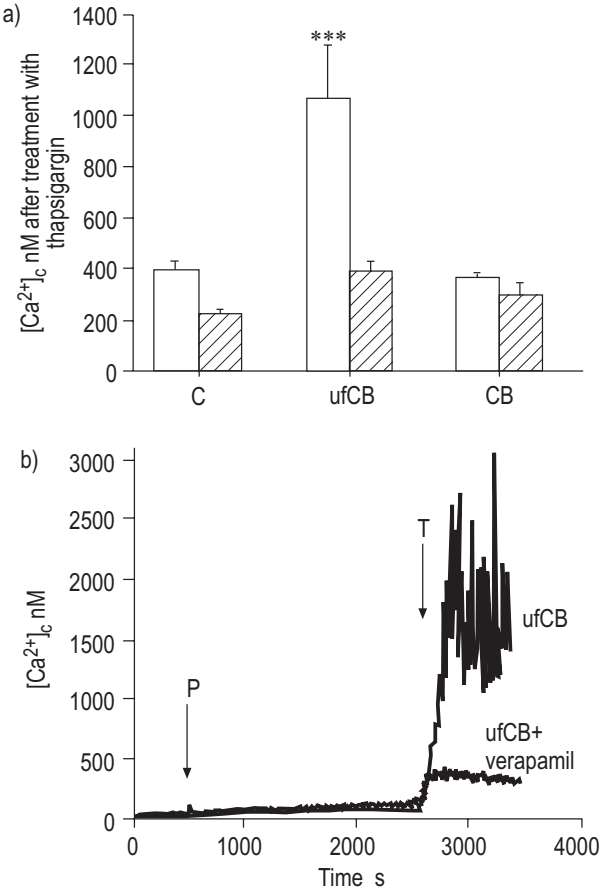

Fig. 1. – a) The effect of particles (P; 66 $\mu g \cdot mL^{-1}$; 2,000 s, 20% foetal calf serum (FCS) on the response of the cytosolic calcium concentration ($[Ca^{2+}]_c$ nM) in MM6 cells on exposure to thapsigargin (T; 100 nM) without (□) and with (▨) verapamil (100 µm). b) Typical traces for MM6 cells treated with ultrafine carbon black alone and with verapamil. The particles were added where indicated. Values are mean±SEM. ***: $p<0.001$. CB: carbon black; uf CB: ultrafine carbon black; C: control.

After exposure to ufCB (66 $\mu g \cdot mL^{-1}$) for 2,000 s the cells were treated with thapsigargin (100 nM final concentration) which increased the $[Ca^{2+}]_c$ to 1067.04±207.35 (n=9; fig. 1), indicating that compared to the control (402.30± 24.27 nM), ufCB induced a 2.6-fold increase in the response to thapsigargin ($p<0.001$).

Treatment with CB (66 $\mu g \cdot mL^{-1}$ for 2,000 s) did not alter either the $[Ca^{2+}]_c$ after 2,000 s (87.92±17.43 compared with 86.84±14.00), nor the response to thapsigargin (fig. 1; $p>0.05$).

The effect of ufCB and CB on the resting $[Ca^{2+}]_c$ and the response to thapsigargin was investigated at varying doses and times. CB did not induce any alteration in the resting $[Ca^{2+}]_c$ or response to thapsigargin at any dose (up to 132 $\mu g \cdot mL^{-1}$ for 2,000 s; $p>0.05$) or time tested (500–2,000 s; $p>0.05$; data not shown).

In contrast ufCB induced a dose dependent increase in the resting $[Ca^{2+}]_c$ so that even at the lowest dose (33 $\mu g \cdot mL^{-1}$) of ufCB studied there was a significant increase in the resting $[Ca^{2+}]_c$ from 86.84±14.00 nM to 112.00±6.18 nM ($p<0.01$). In addition, the response of the MM6 cells to thapsigargin increased as the dose of ufCB increased 33–66 $\mu g \cdot mL^{-1}$ (data not shown; $p<0.05$).

The effects of ufCB on the resting $[Ca^{2+}]_c$ and response to thapsigargin were also found to be time dependent, increasing with increasing time. UfCB (66 μg·mL^{-1}) induced a significant increase in resting $[Ca^{2+}]_c$ ($p<0.01$) and the response to thapsigargin ($p<0.05$) as early as 500 s (8 min). Earlier time points were not investigated. Time points beyond 2,000 s were not investigated because of the large size of the response to thapsigargin again making the calibration of data inaccurate.

Effect of ultrafine carbon black on the response of MM6 cells to thapsigargin

Verapamil. The calcium channel blocker, verapamil, was used to investigate whether the enhanced response to thapsigargin after ufCB treatment could be due to the entry of extracellular Ca^{2+} *via* Ca^{2+} channels. Treatment of the MM6 cells with ufCB (66 μg·mL^{-1}) in the presence of verapamil (100 μM) significantly reduced the response to thapsigargin observed with ufCB alone so that the $[Ca^{2+}]_c$ increased to only 394.78±33.12 nM (n=5) instead of 1067.04±207.35 nM (n=9; fig. 1; $p<0.001$). In accord with the experiments with CB alone, CB in the presence of verapamil did not alter the response of the MM6 cells to thapsigargin ($p>0.05$; fig. 1).

Low-calcium medium and EGTA. In low-Ca^{2+} medium, thapsigargin induced an increase in $[Ca^{2+}]_c$ to 224.30± 14.70 nM (n=5) which declined immediately and exponentially (fig. 2a).

In the low-Ca2+ medium, the response to thapsigargin was not different after treatment with 66 μg·mL^{-1} of either ufCB or CB (194.92±21.29 and 226.38±10.89 nM respectively; fig. 2a). In the presence of ufCB the resting $[Ca^{2+}]_c$ prior to stimulation with thapsigargin was significantly lower than the control (fig. 2b) so that the resting $[Ca^{2+}]_c$ increased at a rate of 0.05±0.01 nM·s^{-1} compared with 0.08±0.01 nM·s^{-1} for the control ($p<0.01$).

Addition of verapamil to the MM6 cells exposed to ufCB in low-Ca^{2+} medium the slope of the resting $[Ca^{2+}]_c$ (0.07±0.012 nM·s^{-1}) was not significantly different from either the control ($p>0.05$) or treatment with verapamil alone in the same conditions (0.06±0.004 nM·s^{-1}; $p>0.05$).

Treatment with CB (66 μg·mL^{-1} for 2,000 s) again did not alter either the rate of increase of the resting $[Ca^{2+}]_c$ (0.09±0.006 nM·s^{-1}) relative to the control (0.08±0.007 nM·s^{-1}; $p>0.05$; fig. 2b) nor the amplitude of the response to thapsigargin ($p>0.05$; fig. 2b).

Repeating the experiments in the presence of the Ca^{2+} chelator EGTA (400 μM) produced similar results in that the response to thapsigargin was not significantly altered from the control (17.6±0.9) by the addition of either ufCB (20.8±3.0) or CB (18 6±0.1; $p>0.05$).

Manganese. Extracellular Mn^{2+} is known to enter cells *via* open Ca^{2+} channels in the plasma membrane. The combination of Mn^{2+} with fura 2 results in a decrease in fluorescence as opposed to the increase observed with Ca^{2+} [23], thus quenching of fura 2 fluorescence by Mn^{2+} was used as an indicator of plasma membrane Ca^{2+} channel opening. Treatment of the MM6 cells with Mn^{2+} (300 μM) in low-Ca^{2+} HBSS induced an immediate decrease in fluorescence from 153.27±10.37 fluorescence units down to 27.61±2.68% of control (fig. 3; n=4). Treatment with

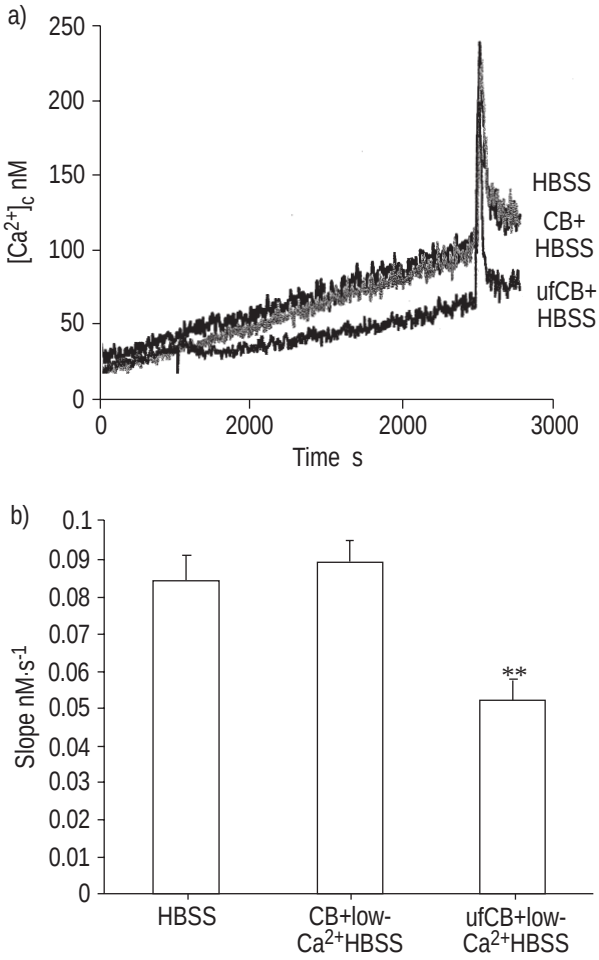

Fig. 2. – a) Typical traces for MM6 cells treated with carbon black (CB), ultrafine carbon black (ufCB) or no particles in the low-Ca^{2+} followed by thapsigargin treatment. b) The effect of CB and ufCB on the slope of the resting cytosolic calcium concentration ($[Ca^{2+}]_c$ nM) in MM6 cells in a low Ca^{2+} Hank's balanced salt solution (HBSS). Values are mean±SEM. **: $p<0.01$.

ufCB in the presence of Mn^{2+} induced a significantly greater decrease in fluorescence to 16.64±1.87% of control ($p<0.01$; n=5; fig. 3). UfCB in the absence of Mn^{2+} deceased the fura 2 fluorescence to 51.18±4.54% of the control (n=4) which was a significantly smaller affect than ufCB or Mn^{2+} alone ($p<0.001$; fig. 3) and was caused by the ability of ufCB to quench the fura 2 fluorescence.

In the presence of verapamil the decrease in fura 2 fluorescence by ufCB in the presence of Mn^{2+} was not significantly different from Mn^{2+} alone ($p>0.05$; fig. 3), providing further evidence to support the activation of Ca^{2+} channels by ufCB.

Effect of the antioxidants, mannitol and nacystelin on the enhanced response to thapsigargin after treatment with ultrafine carbon black

In the presence of the antioxidant mannitol (2 mM) or nacystelin (400 μM) the effect of ufCB on the response to thapsigargin was no longer significantly different from the control ($p>0.05$; fig. 4).

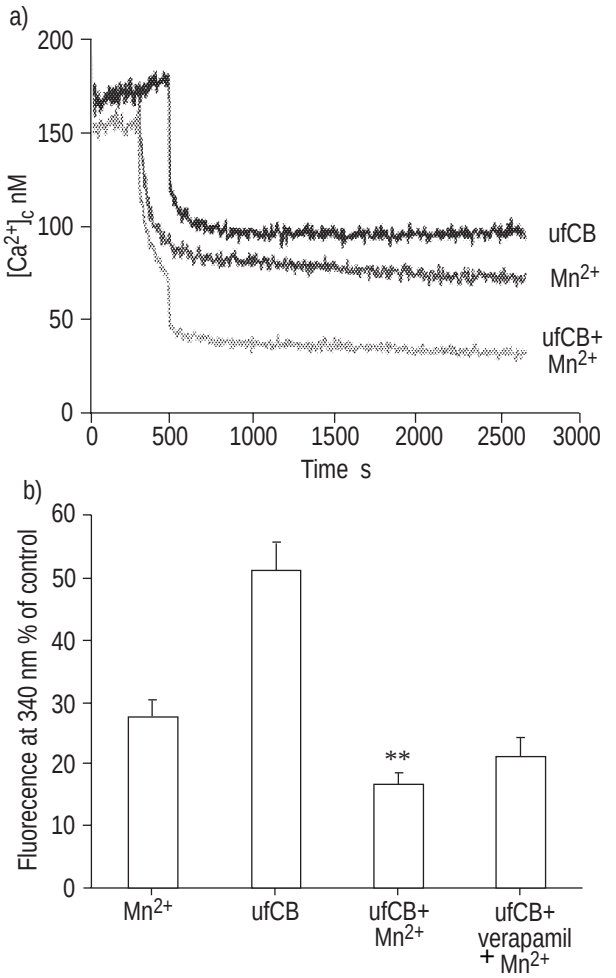

Fig. 3. – a) Typical traces for MM6 cells treated with Mn^{2+} alone, ultrafine carbon black (ufCB) alone and Mn^{2+} in combination with ufCB. b) The effect of ufCB (in the presence and the absence of verapamil (100 μm)), on the quenching of fura 2 fluorescence (340 nm) due to the entry of extracellular Mn^{2+} into the MM6 cells. Values are mean±SEM. **: $p<0.01$. $[Ca^{2+}]_c$: cytosolic Ca^{2+} concentration.

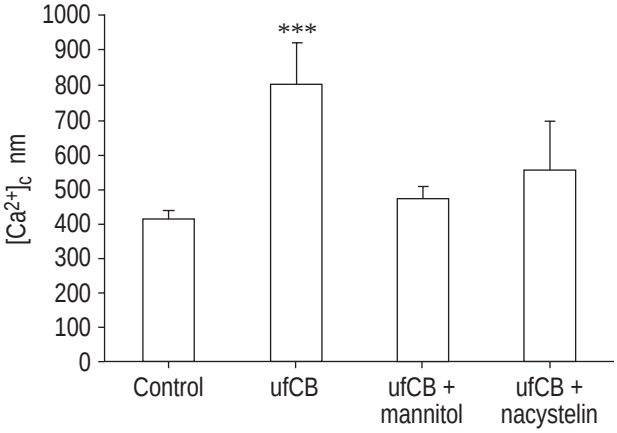

Fig. 4. – The effect of mannitol (2 mM) and nacystelin (400 μm) on the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) observed on exposure to thapsigargin in the presence of ultrafine carbon black (ufCB) (66 $\mu g \cdot mL^{-1}$ for 2,000 s). Values are mean±SEM. ***: $p<0.001$.

Discussion

The measurement of cytosolic Ca^{2+} in the MM6 cell line has provided evidence that ufCB particles stimulate the entry of extracellular Ca^{2+}. UfCB, but not CB induced a 1.6-fold increase in the resting $[Ca^{2+}]_c$ relative to the control. Raised $[Ca^{2+}]_i$ is a well known indicator of cell death [25] and for this reason the viability of the cells was assessed. After treatment of the MM6 cells with ufCB they continued to both exclude trypan blue indicating an intact cell membrane, and to respond to stimulation with thapsigargin. Initially, stimulation with thapsigargin was used as a tool to investigate the possible leak of Ca^{2+} from the ER Ca^{2+} store, indicative of apoptosis [25]. Surprisingly, on exposure to thapsigargin the size of the increase in $[Ca^{2+}]_c$ was more than 2.6-fold greater in the presence of ufCB than in the control or the presence of CB indicating that the ultrafine particles had not induced apoptosis, but instead had enhanced the response to thapsigargin.

Thapsigargin acts by inhibiting the Ca^{2+} - ATPase of the ER, which functions to sequester Ca^{2+} from the cytoplasm so that, in conjunction with Ca^{2+}-ATPases located within the plasma membrane, they can maintain a low $[Ca^{2+}]_c$. This allows Ca^{2+} to act as an efficient intracellular signalling molecule. Inhibition of the ER Ca^{2+}-ATPase leads to a rapid release of the stored Ca^{2+}. The increase in cytosolic Ca^{2+} stimulates the opening of CRAC channels and hence an increase in the Ca^{2+} current across the plasma membrane (I_{CRAC}) [21]. The increased response to thapsigargin, suggests that the ultrafine particles may have increased the I_{CRAC} *via* the CRAC channels in the plasma membrane.

Support for an increased entry of extracellular Ca^{2+} on exposure to ufCB was obtained from three sources. Firstly, the Ca^{2+} channel blocker, verapamil prevented the enhanced response to thapsigargin observed on exposure of the MM6 cells to ufCB. Secondly, experiments conducted in the absence of extracellular Ca^{2+} (low-Ca^{2+}, and EGTA treated cells) indicated that ufCB did not induce any change in the response to thapsigargin indicating that the source of increased intracellular Ca^{2+} was indeed extracellular. In the absence of extracellular Ca^{2+}, Ca^{2+} cannot enter the cells *via* the CRAC channels to replenish the ER Ca^{2+} stores, hence the response to thapsigargin is transient as has been previously published [20]. Thirdly, addition of ufCB to the MM6 cells induced an entry of Mn^{2+} into the cytosol (as indicated by a decrease in fura 2 fluorescence) indicating that the ultrafine particles caused the opening of Ca^{2+} channels in the plasma membrane. Verapamil was also able to partially prevent the reduced slope of the resting $[Ca^{2+}]_c$ as well as inhibiting the entry of Mn^{2+} into the MM6 cells on exposure to the ufCB. Together, these data provide compelling evidence that ufCB stimulates an opening of plasma membrane Ca^{2+} channels; CB does not have this effect.

There are a number of different Ca^{2+} channel types found in nonexcitable cells such as macrophages [26, 27]. The type of Ca^{2+} channel *via* which Ca^{2+} enters on exposure to ufCB particles could only be defined by the use of patch clamp techniques due to a lack of specific well-characterized channel blockers.

The antioxidants mannitol and nacystelin, both significantly inhibited the enhanced response to thapsigargin induced by the ufCB suggesting that reactive oxygen species are involved in the mechanism by which the ultrafine particles induce opening of plasma membrane Ca^{2+} channels in response to thapsigargin. Nacystelin (L-lysine-*N*-acetylcysteinate) is a salt derivative of *N*-acetyl cysteine and L-lysine which has been developed as an antioxidant for topical administration *via* the lungs. The ufCB particles have previously been shown to possess free radical activity [19], hence the free radicals and/or oxidative stress generated by the particles could be responsible for the opening of the Ca^{2+} channels. Nacystelin has been reported to reduce NF-κB activation by asbestos, another pathogenic particle [28].

In order to ascertain whether the changes in Ca^{2+} observed are common to other macrophages, the authors have recently completed a study which shows that ufCB but not CB can significantly increase the resting $[Ca^{2+}]_c$ and the response to thapsigargin in rat bronchoalveolar lavage cells (>80% macrophages; unpublished data), indicating that these results are not confined to a macrophage cell line.

A number of studies have investigated the effects of respirable particles on the Ca^{2+} homeostasis of macrophages or pulmonary cells. Exposure of rat alveolar macrophages to silica (200 $\mu g \cdot mL^{-1}$) induced an increase in $[Ca^{2+}]_c$ within 15 min reaching a maximum increase of 5-fold at 2 h [29]. LIM *et al.* [15] exposed rat alveolar macrophages to relatively high doses of silica (1–5 $mg \cdot mL^{-1}$) and observed an increase in $[Ca^{2+}]_c$ probably due to the initiation of cell death. The authors have previously identified that ufCB can induce greater effects on metabolic competence and intracellular glutathione in A459 cells compared to identical doses of CB [19]. These data highlight the potency of these ultrafine particles in biological systems.

More recently VERONESI *et al.* [30] have investigated the effects of residual oil fly ash (ROFA) and a filtrate of (ROFA) (passed through a 0.2 μm pore filter) on the Ca^{2+} levels in human bronchial epithelial cells (BEAS-2B). Both the complete and the filtered ROFA induced a rapid increase in $[Ca^{2+}]_c$. Inhibition of the Ca^{2+} effects by capsazepine was also associated with reduced levels of interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)α transcription indicating a link between the Ca^{2+} changes and pro-inflammatory gene expression.

Residual oil fly ash [30] and PM10 [4] have both been proposed to exert their biological effects in part through their metal content. As mentioned previously the ufCB contains iron (19 ng·10 mg $particles^{-1}$) which is not detectable in the fine CB. Preliminary data using both leachates of the particles and iron chelators, strongly suggest that the iron component of the ufCB is not responsible for the Ca^{2+} changes reported here, nor the inflammatory effects of ufCB [22].

The data described in the present paper may shed light on the known pathogenicity of ultrafine particles. Ultrafine particles such as CB and TiO2 are known to be more pathogenic than respirable but nonultrafine particles of the same material [1, 31]. At the cellular level the authors have demonstrated that ufCB but not CB impairs metabolic competence in epithelial cells [19] and oxidative stress has been implicated as a key mechanism in ultrafine particle mediated effects [32].

It is widely agreed that certain individuals are highly susceptible to the effects of elevated levels of particulate air pollution [5]. These individuals are suggested to be compromised by existing inflammatory diseases such as chronic obstructive pulmonary disease. Studies are now underway to investigate whether ultrafine particles could "prime" the macrophage cells to hyper-respond to subsequent stimuli such as endotoxin or cytokines which would normally activate the cell through the Ca^{2+} pathway.

In summary, ultrafine carbon black, but not carbon black induced a rapid increase in the resting cytosolic calcium concentration and an increase in the response to thapsigargin putatively through an increase in the size of the calcium release-activated calcium current. The source of this calcium was extracellular and the route of entry was probably *via* calcium channels (possibly voltage-gated). The mechanism by which the ultrafine particles stimulate the opening of the calcium channels appears to involve at least in part, induction of oxidative stress. The potential activation of transcription factors on exposure to ultrafine particles *via* a calcium-mediated mechanism remains the focus of ongoing work. These results show *in vitro* evidence in support of a mechanism whereby ultrafine particles could be more inflammogenic than fine particles. Furthermore, based on calcium changes, the data supports a hypothesis for susceptibility to the effects of particles, in lungs with pre-existing inflammation.

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