Pulmonary inflammation induced by low dose particulate matter exposure in mice

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Running title: Low dose PM causes inflammation and affects mitochondria

Abstract

Air pollution is a ubiquitous problem and comprises gaseous and particulate matter (PM). Epidemiological studies have clearly shown that exposure to PM is associated with impaired lung function and the development of lung diseases such as chronic obstructive pulmonary disease and asthma. To understand the mechanisms involved, animal models are often used. However, the majority of such models represent high levels of exposure and are not representative of the exposure levels in less polluted countries, such as Australia. Therefore, in this study we aimed to determine whether low dose PM_{10} exposure has any detrimental effect on the lungs. Mice were intranasally exposed to saline or traffic-related PM_{10} (1μg or 5μg per day) for three weeks. Bronchoalveolar lavage (BAL) and lung tissue were analysed. PM_{10} at 1μg did not significantly affect inflammatory and mitochondrial markers. At 5μg, PM_{10} exposure increased lymphocytes and macrophages in BAL fluid. Increased NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and IL-1β production occurred following PM_{10} exposure. PM_{10} (5μg) exposure reduced mitochondrial antioxidant manganese superoxide (antioxidant defence system) and mitochondrial fusion marker (OPA-1) whilst increased fission marker (Drp-1). Autophagy marker Light chain 3 microtubule-associated protein (LC3)-II and phosphorylated-AMPK were reduced, and apoptosis marker (Caspase-3) was increased. No significant change of remodelling markers was observed. In conclusion, a sub-chronic low level exposure to PM can have an adverse effect on lung health, which should be taken into consideration for the planning of roads and residential buildings.

Introduction

The World Health Organisation (WHO) air quality model demonstrates that ambient air pollution annually causes 4.2 million deaths, and 91% of the world's population lives in places where air quality exceeds the limits of WHO guidelines. Air pollution causes 1.8 million deaths from lung diseases (1). Forty three percent of chronic obstructive pulmonary diseases (COPD) and 29% of lung cancer deaths are attributable to air pollution (2). PM is the sum of all particles suspended in the air which includes both organic and inorganic particles such as dust, pollens, and vehicle emissions. Respirable PM is thought to be the most detrimental to human health. PM sized equal or below 10 microns (PM_{10}) is capable of entering the lungs, whilst PM sized equal or below 2.5 microns ($PM_{2.5}$) can reach the distal lung segments including alveoli (17) .

In adults, every 5 $\mu g/m^3$ increment of PM exposure is associated with a 39% to 56% increased risk of developing COPD (13). In developed countries such as the UK, traffic related air pollution (TRAP) accounts for 13% of total PM (4). In Sydney Australia, the levels of TRAP are amongst the lowest in the world, accounting for 14% of total PM (5), which often assumed to be safe. However, a study on 65,000 children in Canada found that children exposed to TRAP, even in urban areas with low levels of pollution, had a 25% increased risk of developing asthma by the age of 5 years.

PM is a strong oxidant, with its oxidant capacity regulated by antioxidants such as manganese superoxide dismutase (16). However, in humans, even short-term exposure of PM_{10} increased circulating levels of Interleukins (IL)-1β, IL-6 and TNF-α (28). PM_{10} contains approximately 10^{16} free radicals/g which can increase oxidative stress in human macrophages and lung epithelial cells (8, 29). ROS can induce inflammatory responses via the activation of the

nucleotide-binding domain and leucine-rich repeat protein (NLRP)3 inflammasome, which in-turn cleaves pro-interleukin (IL)-1β into IL-1β. Interestingly, Hirota et al have shown that PM activates the NLRP3 inflammasome resulting in increased IL-1β in bronchial epithelial cells (14).

Mitochondria can be damaged by both oxidative stress and the activation of NLRP3 inflammasome, resulting in reduced capacity to produce ATP. Mitophagy is a quality control process where fission removes damaged mitochondria fragments and fusion merges healthy mitochondrial fragments to regenerate new mitochondria (7), which has been shown to ameliorate inflammatory disorders (23). The impact in low level PM exposure on mitophagy markers has not been reported.

TRAP contains both gaseous and PM components. While the gaseous components are equally toxic as PM, gases dissipate quicker in air than the PMs which can remain airborne for long periods of time. However, most PM / TRAP exposure models used very high PM exposure regimens (e.g. 50 to 200 μg (11, 21)), which are not relevant to the PM/TRAP levels in countries with low levels of air pollution. We hypothesized that exposure to low levels of PM would be detrimental for lung health. Our objective was to establish an environmentally relevant model of TRAP-related PM exposure and to characterise pulmonary changes including inflammasome activation (NLRP 3 and IL-1β), IL-6 production, mitochondrial fission and fusion markers (Optic atrophy (Opa)-1 and dynamin-related protein (Drp)-1), autophagy markers and fibrotic markers (fibronectin, collagen III and transforming growth factor beta 1 (TGFβ1)).

Materials and Methods

PM collection

Twenty-four-hour integrated PM_{10} were collected through a 47-mm Teflon (Pall Life Sciences, Ann Arbor, MI) and pre-fired (800 °C, 3 hr) 47-mm quartz-fibre filters (Whatman Inc., Clifton, NJ) from a busy roadside in Hong Kong (114,000 vehicles per day) with URG PM samplers (URG-2000-30EH) in the summer (24th June to 11th July, 2017) with a flow rate of 8 L/min at each channel. Filter preparation (e.g. equilibrated for 24 hr at 25 °C and relative humidity of 40% before and after sampling) and gravimetric analysis were conducted in a high-efficiency particulate absorption clean room (ISO 14644 Class 7) at The Hong Kong Polytechnic University. All filters were stored at -20 °C and in dark prior to the analysis. PM was extracted in 90% ethanol with 5 minutes of sonication, followed by freeze drying overnight.

PM analysis

Energy-dispersive x-ray fluorescence spectrometry (PANalytical Epsilon 5) was used to determine concentrations of Al, Si, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ba and Pb. Each sample was analysed for 30 min. Thin-film standards were used for calibration (MicroMatter, Arlington, USA) (34). All reported chemical concentrations were corrected for field blanks, and duplicated samples were analyzed for quality assurance.

Ion chromatography (IC) for water-soluble inorganic ions analysis. One quarter of the filter was extracted with 10 mL of distilled deionized water and the extract underwent IC (Dionex DX-600) analysis (IonPac CS12A and AS14A columns) Six species were analysed as previously described (36). Analysis of organic carbon and elemental carbon were by thermal optical reflectance (TOR) technique on a thermal/optical carbon analyser (DRI Model 2001, Atmoslytic Inc., Calabasas, CA as described in Pathak et al (22).

In vivo PM exposure.

Animal experiments were approved by the Animal Care and Ethics committee at the University of Technology Sydney (ACEC#ETH16-0886). Male Balb/c mice (6 weeks, Animal Resources Centre, Perth, Australia) were housed at 20 ± 2 ^{o}C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h) with *ad libitum* access to standard laboratory chow and water. After the acclimatisation period, mice were assigned to 3 groups (n =10) which were exposed to either particulate matter with 1µg (PM_{10}(1µg)) or 5µg (PM_{10}(5µg)) or saline as control (SHAM). In urban Sydney, the average PM_{10} levels are 17 µg/m^3, equating to a daily human exposure of 181µg (3). Based on the breathing volumes, mice should be exposed to around 5µg/day to reflect air pollution levels in Sydney. Mice were exposed intranasally by instillation of 40µl of saline or saline resuspended PM_{10} daily for three weeks.

At the endpoint, the animals were sacrificed via cardiac puncture after deep anaesthesia (3% isoflurane). Lungs were perfused with phosphate buffered saline to obtain bronchoalveolar lavage (BAL) fluid. Lungs were then harvested, snap frozen and stored at -80^{o}C for protein analysis. Anthropometry measurements were done following dissection and measurement on a microbalance.

BAL analysis.

The BAL cells evaluated by Diff-Quik staining (Polyscience Inc, Taipei, Taiwan). Differential cell counts were performed for macrophages, lymphocytes, eosinophils and neutrophils.

Western blotting.

Lung tissue homogenates (20μg) were analysed using standard techniques, as described previously (9). Antibodies were purchased from Cell Signaling Technology, USA: IL-1β and IL-6 (1:1000); Caspase-3, p-Akt, Akt, p- AMP-activated protein kinase (AMPK), AMPK, light chain 3 microtuble-associated protein (LC)3A/B-I/II (1:2000); from Novus Biotechnology, USA: Drp-1, Opa-1 (1:2000) and Collagen-III (1:1000); from Millipore, USA: MnSOD (1:2000,); from Sigma-Aldrich, USA Fibronectin (1:2000); and R&D systems, USA: TGF-β1 (1:500).

Mitochondrial DNA copy number.

mtDNA was measured using qPCR on DNA as we have previously published (25, 26).

Statistical methods.

The data conformed to the normal distribution and differences between groups were analysed using one-way ANOVA followed by a Bonferroni post-hoc tests. $P<0.05$ was considered significant.

Results

PM characterisation

The main components of the PM were organic carbons. Sulphate, elemental carbon, chloride and nitrate were the other components in abundance in the PM sample. Traces of other substances such as titanium, manganese, lead, chromium and nickel were also detected, see Table 1.

Anthropometry markers

Weight gain was used as a generic indicator of health status. As shown in Table 2, body weight was not affected by PM exposure (Table 2). However, PM_{10} (5ug)-exposed animals had significantly more retroperitoneal fat mass compared to the SHAM group ($p<0.05$). There were no significant changes in liver or muscle weights.

Bronchoalveolar (BAL) cell count

PM_{10} (5μg) exposure increased leukocyte counts in BAL fluid ($P<0.01$, PM_{10} (5μg) vs SHAM, Figure 1A), as well as lymphocytes and macrophages (both $P<0.01$ vs SHAM, Figure 1A, B). There were no neutrophils or eosinophils observed.

Lung Inflammation

NLRP 3 and IL-1β were increased in the PM_{10} (5μg) group compared to the SHAM group ($P<0.05$, Figure 1D/E), but not IL-6 (Figure 1F).

Markers of matrix remodelling

Protein levels of fibronectin, TGF-β1 and collagen-III were not changed in any of the PM groups compared to the SHAM group (Figure 1G-I).

Mitochondrial antioxidant, mitophagy markers and mitochondrial DNA copy number

PM_{10} (5μg) exposure significantly increased mitochondrial fission protein Drp-1 ($P<0.05$, PM_{10} (5μg) vs SHAM, Figure 2A) and reduced mitochondrial fusion protein OPA-1 and the antioxidant MnSOD levels (both $P<0.05$, PM_{10} (5μg) vs SHAM, Figure 2B/C). Mitochondrial DNA copy number was not changed between SHAM and PM_{10} (5μg) (Figure 2D).

Autophagy and apoptosis

Autophagy marker LC3A/B-II, LC3A/B-II to I ratio were reduced in PM_{10} (5μg) compared to SHAM ($P<0.05$, Figure 2E/F). Apoptotic marker Caspase-3 was increased in the PM_{10} (5μg) group compared to the SHAM group ($P<0.05$, Figure 2G). The upstream marker of autophagy, p-AMPK and p-AMPK to AMPK ratio were reduced by the exposure to PM_{10} (5μg) compared to the SHAM exposure ($P<0.05$ vs SHAM, Figure 2K/M). Akt and AMPK protein levels were increased in the PM_{10} (5μg) group compared to the SHAM group ($P<0.05$ vs SHAM, Figure 2I/L), but there were no changes in p-Akt protein levels and p-Akt to Akt ratio by PM_{10} exposure (Figure 2I/J).

Discussion

We found that the exposure to low levels of traffic related PM_{10} induced marked pulmonary activation of NLRP3 inflammasome, and inflammation, as well as reduced mitochondrial antioxidants, and impaired mitophagy capacity.

PM_{10} exposure for three weeks did not affect the overall wellbeing of the mice reflected by body weight, suggesting low toxicity. However, fat mass was increased following the exposure to 5μg of PM_{10}, consistent with other human and mouse studies (27, 31).

We found increased lymphocytes and macrophages, which has also been observed with high dose PM exposure (8). However, PM_{10} (5μg) did not induce eosinophilic or neutrophilic inflammation. Increased IL-1β was accompanied by NLRP3 inflammasome activation as expected. Zheng et al (37) also found that 3 weeks exposure to 50μg of $PM_{2.5}$ daily increased IL-1β and TGF-β1 levels in BAL. Inflammasome activation has been observed in asthma, COPD and during pulmonary inflammation (10, 18, 35), suggesting that continuous exposure to even low level of PM may increase the susceptibility to these conditions.

Mitochondrial dysfunction is associated with various pulmonary diseases. COPD patients have mitochondrial fragmentation through an increase in Drp-1. In-vitro prolonged cigarette smoke exposure increased mitochondrial fission (6, 15). Damaged mitochondria increase oxidative stress which can consume the antioxidative MnSOD. Our study shows that 5μg of PM reduced MnSOD, suggesting reduced antioxidant capacity. Mitochondrial DNA copy number was unaffected, suggesting mitochondrial biogenesis was not changed by PM in this model. The reduction in LC3A/B-II protein in the PM_{10} (5μg) group indicates that there was reduced capacity of autophagy which can increase apoptosis. This was confirmed with the increased protein levels of caspase-3 in our study.

Activated AMPK was reduced by PM_{10} exposure. AMPK is a stress sensor which is crucial for maintaining intracellular homeostasis during oxidative stress and importantly, AMPK deficient mice have increased progression of COPD (19). AMPK typically supresses Akt, but we found no change in Akt levels, suggesting dysregulation of AMPK/Akt signalling. In our study we found PM reduced AMPK activation with reduced autophagy, however *in-vitro* studies have found PM increases AMPK and autophagy. We postulate that such differences are related to the 10-20 times higher dose of PM used *in-vitro* which induce cell death, in-addition to activating AMPK and autophagy (20, 30, 32). The *in-vitro* response is consistent with the notion that autophagy generally acts to keep cells alive, and is upregulated in response to stress (for a review see (12)). Differences may also occur due to PM processing for *in-vitro* studies in which steam sterilisation to remove LPS may also remove other PM components. Interestingly LPS inhibits AMPK activation (33).

Inflammasome activation by asbestos or crystalline silica is strongly associated with the development of lung fibrosis (24). However, in this study, exposure to a low level of PM did not induce fibrosis. The negative findings are most likely attributable to the low PM dose and the short duration of this study.

This study has several limitations. PM_{10} composition varies by generation source, and as such future studies need to compare different types of PM. We did not assess endotoxin levels in PM which are likely to influence the proinflammatory capacity of the PM. The lung tissues were not fixed to assess any histological changes or mitochondrial morphology, which need to be addressed in future studies.

In conclusion, this study shows that the exposure to low levels of roadside PM has detrimental effects on lung health. As such people living alongside major traffic corridors need to be aware of the potential adverse effects on their respiratory health. Our results also have implications for government agencies responsible for urban planning.

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Figure Legends

Figure 1. Leukocytes counts bronchoalveolar lavage (A-C). Lung protein levels of NLRP3 (D), IL-1β (E), IL-6 (F), fibronectin (G), TGF-β1 (H) and collagen-III (I) in Sham, particulate matter $(PM)_{10}$ (1μg) and PM_{10} (5μg) groups. Results are expressed as mean ± SEM, n = 8-10 (one-way ANOVA followed by Bonferroni post hoc test). *p<0.05, **p<0.01, compared with SHAM; #P<0.05, ##p<0.01, compared with PM_{10} (1μg).

Figure 2. Lung mitochondrial protein levels of Drp-1(A), Opa-1(B), MnSOD (C), Mitochondrial DNA copy number (D) , Lung protein levels of LC3A/B-II (E), LC3A/B-II to I ratio (F), Caspase-3 (G), p-Akt (H), Akt (I), p-Akt/Akt ratio (J), p-AMPK (K), AMPK (L) and p-AMPK to AMPK ratio (M) in Sham, PM_{10} (1μg) and PM_{10} (5μg) groups. Results are expressed as mean ± SEM, n=5-8. (one-way ANOVA with Bonferroni tests). *P<0.05 compared to SHAM. **P<0.01 compared to SHAM, #P<0.05, compared to PM_{10} (1μg). Akt, protein kinase 3; AMPK, 5' adenosine monophosphate-activated protein kinase; Drp-1, dynamin related protein 1; LC3A/B, Light chain 3 microtubule-associated protein A/B; MnSOD, manganese superoxide dismutase; Opa-1, optic atrophy 1; PM, particulate matter.

418 **Chemical components of PM**

419 **Table 1. Chemical characteristic of PM_{10}**

	µg/m^3		µg/m^3
PM_{10} mass	22.61±1.26	**Ammonium**	0.16±0.03
Organic Carbon (OC)	4.19±0.20	**Barium**	0.08±0.003
Sulfate	4.00±0.34	**Zinc**	0.08±0.01
Elemental Carbon (EC)	3.26±0.17	**Copper**	0.04±0.03
Chloride	2.52±0.41	**Titanium**	0.02±0.004
Nitrate	1.92±0.13	**Manganese**	0.02±0.002
Iron	0.85±0.04	**Lead**	0.02±0.002
Calcium	0.43±0.03	**Vanadium**	0.01±0.002
Silicon	0.35±0.02	**Chromium**	0.01±0.001
Aluminium	0.17±0.02	**Nickel**	0.01±0.001

420 Results are expressed as mean ± SEM. Data showing different components inside the traffic
421 related air pollutants (n=10).
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424 **Table 2. The effects of PM_{10} exposure on anthropometry markers**

	SHAM	**PM_{10} (1µg)**	**PM_{10} (5ug)**
Body Weight	22.39±0.31	22.26±0.36	22.13±0.37
Liver (g)	1.26±0.045	1.21±0.037	1.15±0.037
Liver %	5.62±0.0015	5.47±0.0011	5.21±0.0015
Muscle (g)	0.073±0.0024	0.075±0.0023	0.072±0.0032
Muscle %	0.33±0.00013	0.34±0.00011	0.33±0.00019
Retroperitoneal fat weight (g)	0.077±0.0037	0.109±0.014	0.12±0.012*
Retroperitoneal fat %	0.34±0.00016	0.50±0.00064	0.55±0.00052*
Glucose (mM)	9.13±1.14	9.6±1.07	9.27±1.1

Results are expressed as mean ± SEM, n = 10. Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. *$p<0.05$, compared with SHAM. PM_{10}: particulate matter.

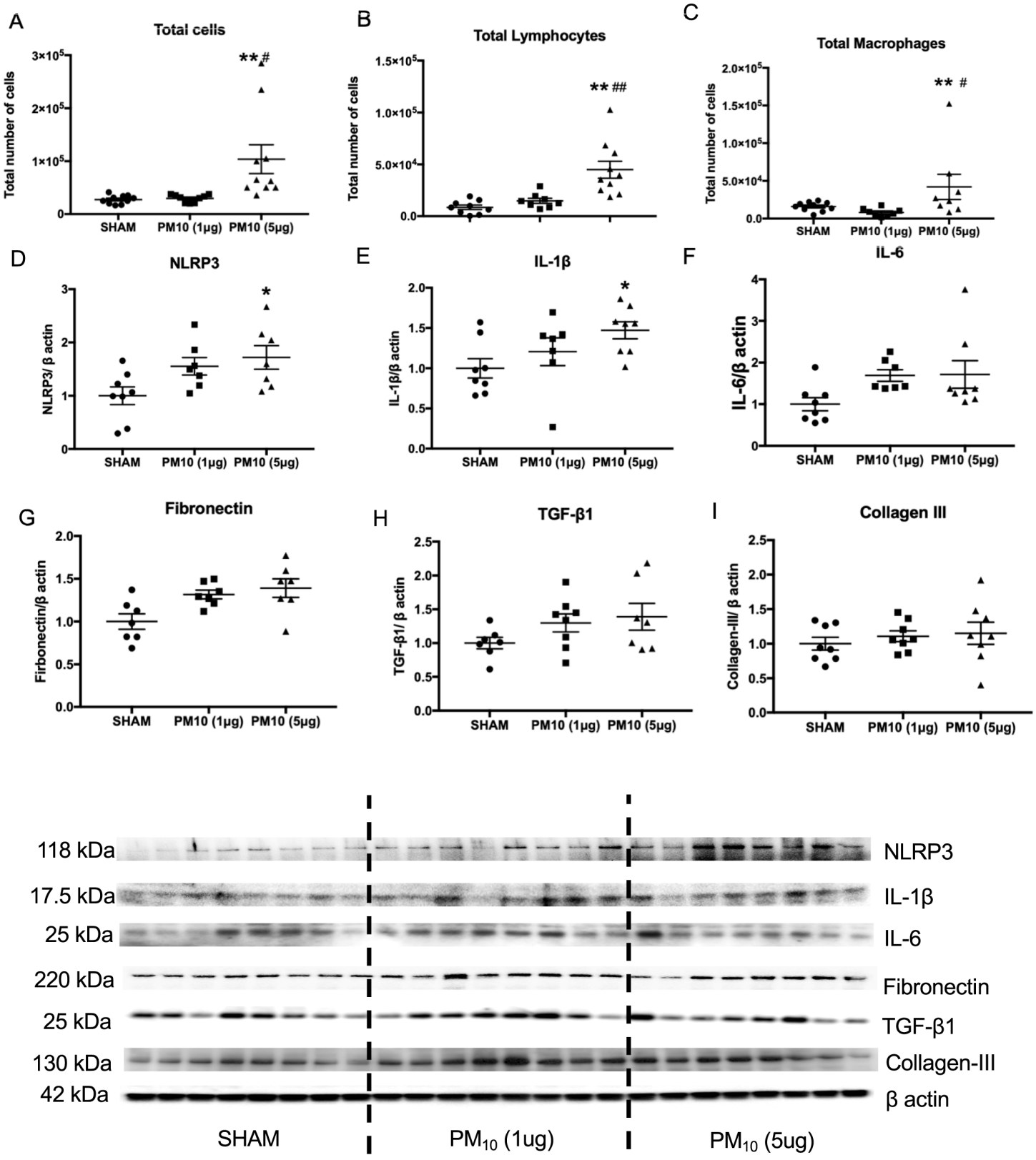

Figure 1.

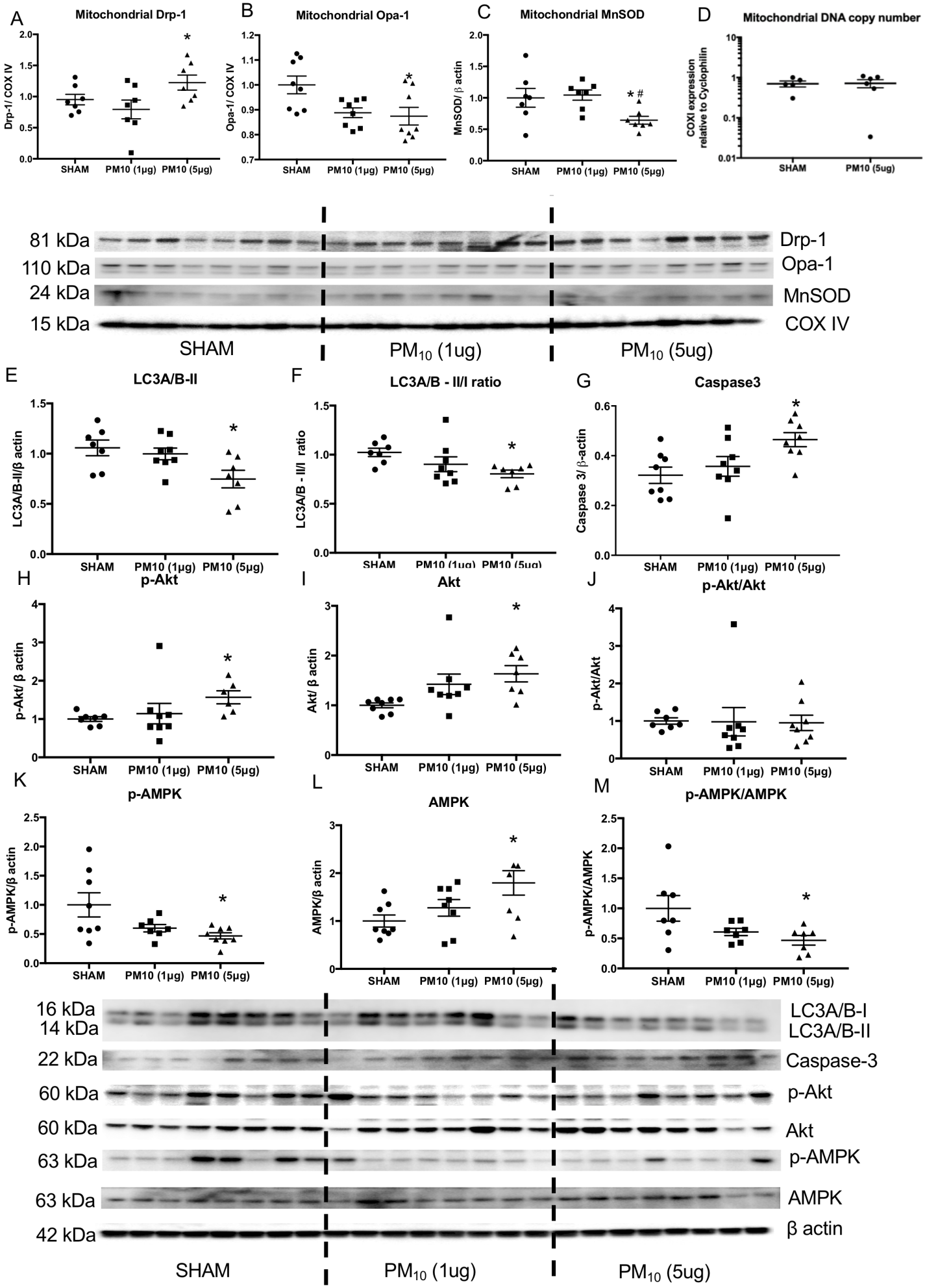

Figure 2.