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Aging attenuates redox adaptive homeostasis and proteostasis in female mice exposed to traffic-derived nanoparticles ('vehicular smog')

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ABSTRACT

Environmental toxicants are catalysts for protein damage, aggregation, and the aging process. Fortunately, evolution selected adaptive homeostasis as a system to mitigate such damage by expanding the normal capacity to cope with toxic stresses. Little is known about the subcellular degradative responses to proteins oxidatively damaged by air pollution. To better understand the impact of environmental toxicants upon the adaptive homeostatic response, female C57BL/6 mice were exposed for 10 weeks to filtered air or reaerosolized vehicular-derived nano-scale particulate matter (nPM), at which point tissues from young (6 month) and middle-aged (21 month) mice were studied. We found significant increases of proteolytic capacity in lung, liver, and heart. Up to two-fold increases were seen in the 20S Proteasome, the Immunoproteasome, the mitochondrial Lon protease, and NF-E2-related factor 2 (Nrf2), a major transcriptional factor for these and other stress-responsive genes. The responses were equivalent in all organs, despite the indirect input of inhaled particles to heart and liver which are downstream of lung. To our knowledge, this is the first exploration of proteostatic responses to oxidative damage by air pollution. Although, middle-aged mice had higher basal levels, their Nrf2-responsive-genes exhibited no response to nanoparticulate exposure. We also found a parallel age-associated rise in the Nrf2 transcriptional inhibitors, Bach1 and c-Myc which appear to attenuate adaptive responses in older mammals, possibly explaining the 'age-ceiling effect.' This report extends prior findings in male mice by demonstrating the involvement of proteolytic responses to traffic-related air pollution in lung, liver, and heart of female mice, with an age-dependent loss of adaptive homeostasis.

1. Introduction

Chronic exposure to traffic-related air pollution (TRAP) particulate matter can have deleterious health consequences, evident in high-smog environments. Even more troubling is the impact of daily low level exposure to a subset of TRAP-derived particles ($PM_{2.5}$) [1] upon human health. In the Women's Health Initiative Memory Study (WHIMS) cohort of 65,000 postmenopausal women, chronic exposure to air

Abbreviations: nPM / $PM_{0.2}$, Nano-Scale Particulate Matter / Particulate Matter < 0.2 μm diameter; $PM_{2.5}$, Particulate Matter < 2.5 μm diameter; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; Bach1, BTB Domain And CNC Homolog 1; c-Myc, MYC proto-oncogene; TRAP, Traffic-related air pollution; WHIMS, Women's Health Initiative Memory Study; Keap1, Kelch Like ECH Associated Protein 1; ARE, Antioxidant Response Element; EpRE, Electrophile Response Element; HO-1, Heme Oxygenase-1; NQO1, NAD(P)H Quinone Dehydrogenase 1; SOD1, Superoxide Dismutase 1; *C. elegans*, Caenorhabditis elegans; *D. melanogaster*, Drosophila melanogaster; [^{3}H]Hb, Tritium-tagged hemoglobin; [^{3}H]$Hb_{oxdized}$, Tritium-tagged oxidized-hemoglobin; ATP, Adenosine Triphosphate; LMP2, Proteasome Subunit Beta-1i; LMP7, Proteasome Subunit Beta-5i; GCLC, Glutamate-Cysteine Ligase Catalytic Subunit; GCLM, Glutamate-Cysteine Ligase Modifier Subunit; CSC, Cigarette smoke condensate; BCA, Bicinchoninic acid; Z-LLG-AMC, Z-Leu-Leu-Glu-AMC; Z-ARR-AMC, Z-Ala-Arg-Arg-AMC; Suc-LLVY-AMC, Suc-Leu-Leu-Val-Tyr-AMC; Ac-PAL-AMC, Ac-Pro-Ala-Leu-AMC; Ac-ANW-AMC, Ac-Ala-Asn-Trp-AMC; AMC, 7-amino-4-methylcoumarin; H_2O_2, Hydrogen peroxide; BSA, Bovine Serum Albumin

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pollution, consisting of $PM_{2.5}$, which is above EPA standards, increased the risk of cardiovascular disease [2]. Similarly, exposure to TRAP-derived particles promotes inflammation in multiple tissues [3,4], including the small airways, further exacerbating asthma and chronic bronchitis development [5]. Moreover, a WHIMS subset had nearly 2-fold higher dementia from excess $PM_{2.5}$ exposure [6]. Additionally, chronic exposure to TRAP particulate matter leads to cumulative and harmful changes in healthy older adults, resulting in increased mortality [7]. Yet, our mechanistic understanding behind TRAP-promoted morbidity and mortality is limited. Due to the cellular stress response being crucial for cellular homeostasis, it is paramount we understand how this pathway is impacted following particulate exposure. Here, we address the interaction between aging and a relatively unexplored subfraction of ambient $PM_{2.5}$, termed nanoscale particulate matter (nPM, $PM_{<0.2}$), which lacks black carbon and water-insoluble organics, with particle diameter of 0.2 μm and smaller (nPM, $PM_{<0.2}$) [8]. Prior work has identified this subset of $PM_{2.5}$-derived particles as having greater cytotoxicity [9] and to act as a pro-inflammatory inducer [8] compared to larger PM.

Cellular stress response pathways are evolutionarily-conserved mechanisms that attenuate and remove cellular damage to lipids, proteins, and DNA [10]. Dysregulated protein homeostasis and adaptive stress responses are considered 2 of the 7 contributing factors of aging [11]. A major enzyme of the proteostasis pathway is the 20S proteasome and is the primary means for protein turnover within the cell [12]. It consists of four rings comprised of 2-outer alpha rings, necessary for substrate-recognition, and two inner beta rings, necessary for catalytic activity: caspase-like (β_1), trypsin-like (β_2), and chymotrypsin-like (β_5) activity [12]. Upon activation of the adaptive stress-protective response [13], an immediate pool of 20S proteasome becomes available (~ 1 h), due to HSP70-mediated sequestering of the highly oxidant-inactivated 19S regulatory caps and subsequent dissociation of the 26S proteasome [14,15]. Concurrently, Nrf2-mediated transcriptional up-regulation of de-novo 20S proteasome subunits, further increases the available pool of 20S proteasome (within 16 h) [16]. Thus the homeostatic role of Nrf2-activation of the 20S proteasome is crucial for protein quality control.

A key lynchpin of the adaptive stress-protective pathway is NF-E2-related factor 2 (Nrf2), a crucial master transcriptional regulator of many Phase II detoxification and stress responsive enzymes. Nuclear translocation of Nrf2 is induced by diverse stressors, including exogenous oxidants [16,17], heavy metals [18], and nPM exposure [19]. Additionally, ultrafine particle exposure (< 0.2 μm) increased Nrf2-targeted stress responsive genes [20]. Under non-stressed conditions, Nrf2 is continuously synthesized and degraded by a mechanism dependent upon its interaction with Keap1, which facilitates its ubiquitination. The ubiquitinated Nrf2 is then degraded by the 26S Proteasome [21]. This limits Nrf2 translocation into the nucleus. Electrophilic modifications of Keap1, which occurs continuously, but is increased most during oxidative stress, prevents its binding to Nrf2. In turn, enabling newly synthesized Nrf2 to escape degradation and to promote its nuclear accumulation. Once in the nucleus, Nrf2 binds to electrophile response elements (EpRE, which are also known by the misnomer, antioxidant response elements or ARE) in the promoter regions of Phase II genes (such as HO-1 [22], GstDs [23], NQO1 [24] and stress-responsive proteins, including the 20S Proteasome [25] and SOD1 [26]).

During aging, basal Nrf2 activation is highly tissue-dependent. Moreover, there is high age-dependent variability in Nrf2 levels pervasive in the literature. Some studies suggest Nrf2 levels decrease in rat liver from 20 month old animals [27,28], whereas, other studies report Nrf2 levels are suggested to rise in aged tissue [29]. Indeed, Nrf2 variability in aging is evident when assessing the age-associated impact upon the unstressed levels of Phase II detoxification genes, which are inconsistent in regards to direction and the extent of change with age [30–32].

Consequently, the 20S proteasome is negatively impacted by the dysregulation of Nrf2 transcriptional activity. Numerous studies have assessed the age-associated impact upon 20S expression and activity, but without conclusive findings. Variation arises between tissues [33–36], species [37,38], and the sexes [39–41]. In contrast, one age-dependent trend remains consistent: the age-related inability to activate Nrf2-dependent stress-responsive genes [24]. In other words, the basal expression of Nrf2-dependent genes varies markedly in aging, but the ability to respond to oxidative challenges consistently declines with age.

Prior work has shown that mild and transient exposure to low doses of an oxidant (such as hydrogen peroxide) is capable of activating the Nrf2-mediated pathway without causing undo cellular harm [42,43]: a process dubbed ‘adaptive homeostasis’ [10]. This process is demonstrated in cell culture [44] and the model organisms *C. elegans* [45] and *D. melanogaster* [39,46–48]. In order to better understand the conserved role of the adaptive homeostatic response, requires understanding if similar changes arise in a mammalian system. One such physiologically relevant approach is to expose mice (at different ages) to sub-lethal amounts of traffic-derived nanoparticulate matter (nPM) [19]. In turn, improving our understanding of the adaptive homeostatic stress response, especially in the context of environmental toxicants.

A highly relevant aspect of the present study is the exploration of sex-dependent differences in a mammalian model. Earlier studies have demonstrated a clear sexual dimorphism in stress protective responses and activation of the adaptive response in lower organisms [49,50]. Moreover, sex is a strong predictor of lifespan in mammals, with females typically outliving males [51,52]. However, actually testing whether sex differences in the regulation of the adaptive response is conserved in a mammalian model has been limited. The present study addresses the exploration of the female-specific response to short-term, sub-lethal amounts of traffic-derived nanoporticulate matter. Although our study is not a direct, side-by-side comparison of males and females, our findings do offer an important comparison of females studied under identical experimental protocols as those used in our previous report of male adaptive responses [19]. The present work also explores the consequences of sub-lethal exposure to environmental particles (‘smog’), which has been previously implicated in accelerating neurological pathologies [53,54], including Alzheimer's disease [55] which is a highly female-favored disease [6], associated with the loss of proteostasis and Nrf2 regulation [56,57].

The majority of the work associated with our understanding of the adaptive homeostatic stress response has centered upon changes related to Nrf2. Yet, other regulators, which directly interact with Nrf2, are worth closer examination. One such is c-Myc, primarily known for its role as a proto-oncogene [58], with more recent findings indicating its interaction with Nrf2 [59]. Specifically, c-Myc silencing leads to the prolonged half-life of nuclear Nrf2, in both basal and induced conditions [59], suggesting c-Myc may play a role in nuclear Nrf2 turnover. The constitutively expressed Bach1 acts as an Nrf2 nuclear antagonist, which also binds to EpRE and thereby competes with Nrf2-mediated gene expression [60,61]. However, during oxidative stress, Nrf2 is imported into the nucleus, while Bach1 dissociates from EpREs. This suggests that Bach1's role as a suppressor may be a necessary means of dampening the Nrf2 response, and potentially serving as an ‘off-switch’ for the adaptive homeostatic response.

Lastly, it is notable that the majority of prior work on aging relies upon comparing a young organism to an aged organism. Though relevant in furthering our understanding about the impact of aging upon the stress response, we have far less understanding of the transformative period that arises during middle-age. Although it is important to highlight important work done in this area over the past 25 years [19,24,62–64]. Our more recent studies comparing nPM exposure in young (6 month) and middle-age (21 month) male mice found depression of Nrf2 activation and higher levels of its transcriptional repressors, Bach1 and c-Myc in aging [19,24], indicating the deleterious consequences of aging may begin at a much earlier time frame than

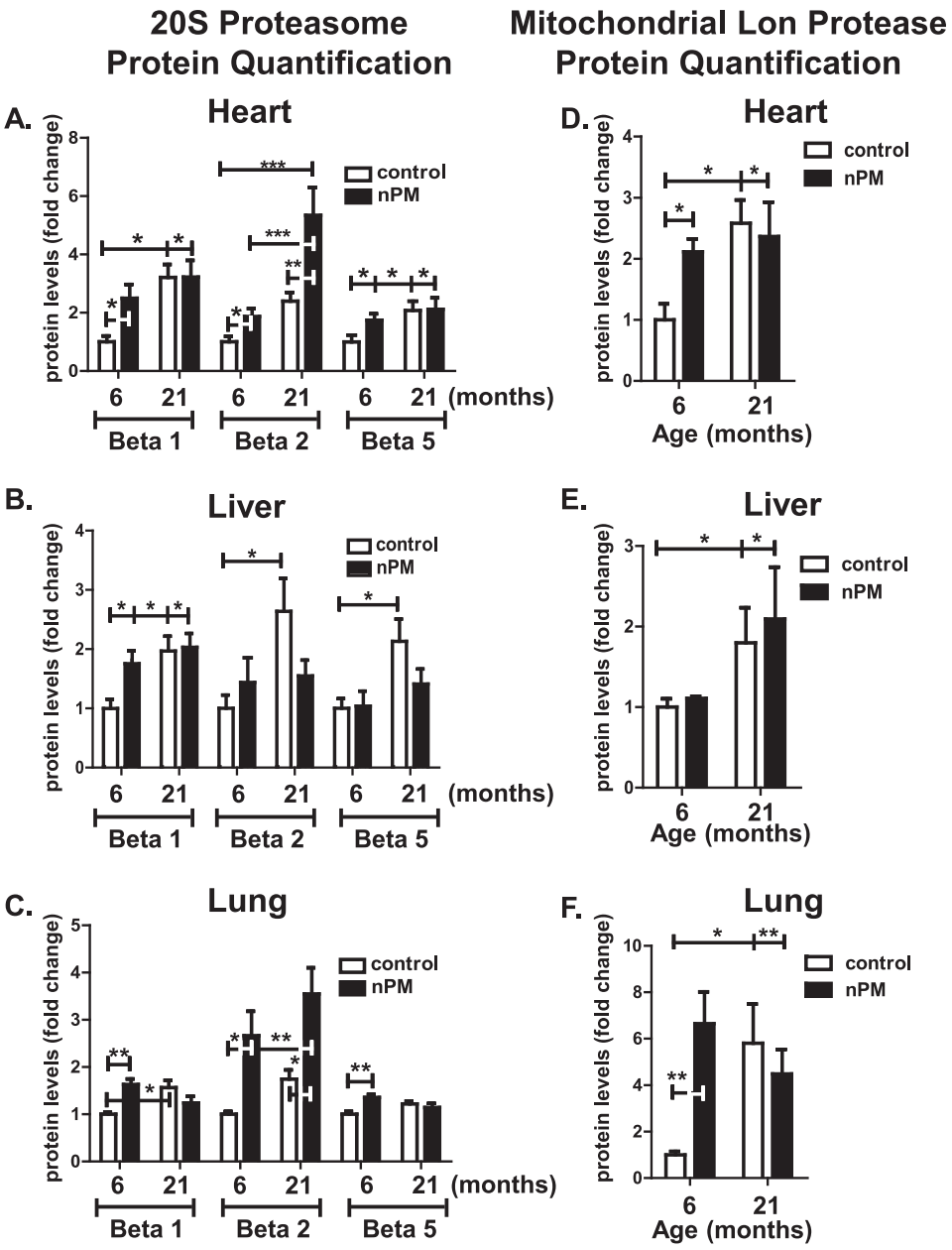

Fig. 1. 20S proteasome subunits and mitochondrial Lon protease expression change with age and nPM exposure. 6 month and 21 month old female mice were exposed to filtered (white) or nPM (black) air, prior to tissue collection. Protein quantification of 20S Proteasome beta subunits. (A) Heart. (B) Liver. (C) Lung. Protein quantification of mitochondrial Lon protease (D) Heart. (E) Liver. (F) Lung. Images were quantified using ImageJ and the levels presented in the bar graph. Error bars denote standard errors of the mean (S.E.M) values, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to the 6 month control using one-way ANOVA (6 animals per treatment group: 6 animals for 6 month controls, 6 animals for 6 month exposed to nPM air, 6 animals for 21 month controls, and 6 animals for 21 month exposed to nPM air). Western blots are presented in Supporting Figs. 1 and 2.

previously proposed.

In the present study, we sought to address the age-associated and tissue-specific changes in 6 month versus 21month old female C57BL/6 mice exposed to either filtered air or sub-lethal amounts of reaerosolized nPM. A novelty of the present study is assessment in age and nPM-dependent changes in the amount and activity of the 20S proteasome. We also addressed the impact of nPM exposure upon the Immunoproteasome, which has been previously linked to inflammation. Lastly, we explored changes in Nrf2 and its regulators.

2. Results

2.1. Tissue specific differences in 20S proteasome subunits and mitochondrial Lon protease protein levels following nPM exposure

The 20S Proteasome and mitochondrial Lon protease are crucial for the clearance of oxidized proteins. We sought to assess the impact following sub-lethal nPM exposure in young (6 month) and middle-aged (21 month) female mice (Fig. 1). 20S β-subunits exhibited higher levels in a tissue-dependent manner (1–2 fold change) in 6 month nPM exposed females compared to 6 month controls (Fig. 1A-C & Supporting Fig. 1A-C). Tissue from aged control animals showed higher β-subunit amounts. However, nPM exposure failed to further increase 20S subunit levels in 21 month mice. Thus, although the basal levels of the 20S Proteasome subunits were higher in older animals, nPM exposure failed to elicit an adaptive response in 21 month mice.

Similarly, the mitochondrial Lon protease revealed tissue-specific differences in protein levels. Heart and lung tissue from 6 month nPM exposed females had higher amounts of Lon, compared to tissue from age-matched controls (Fig., 1D,F & Supporting Fig. 2A,C). In contrast, liver tissue from 6 month controls or nPM exposed females showed no change (Fig. 1E & Supporting Fig. 2B). Basal levels of Lon were higher with age in all three tissues. In addition, nPM exposure had no impact on Lon levels in 21 month nPM exposed females, as tissues showed the same amount of Lon between 21 month controls and nPM treated (Fig. 1E-F).

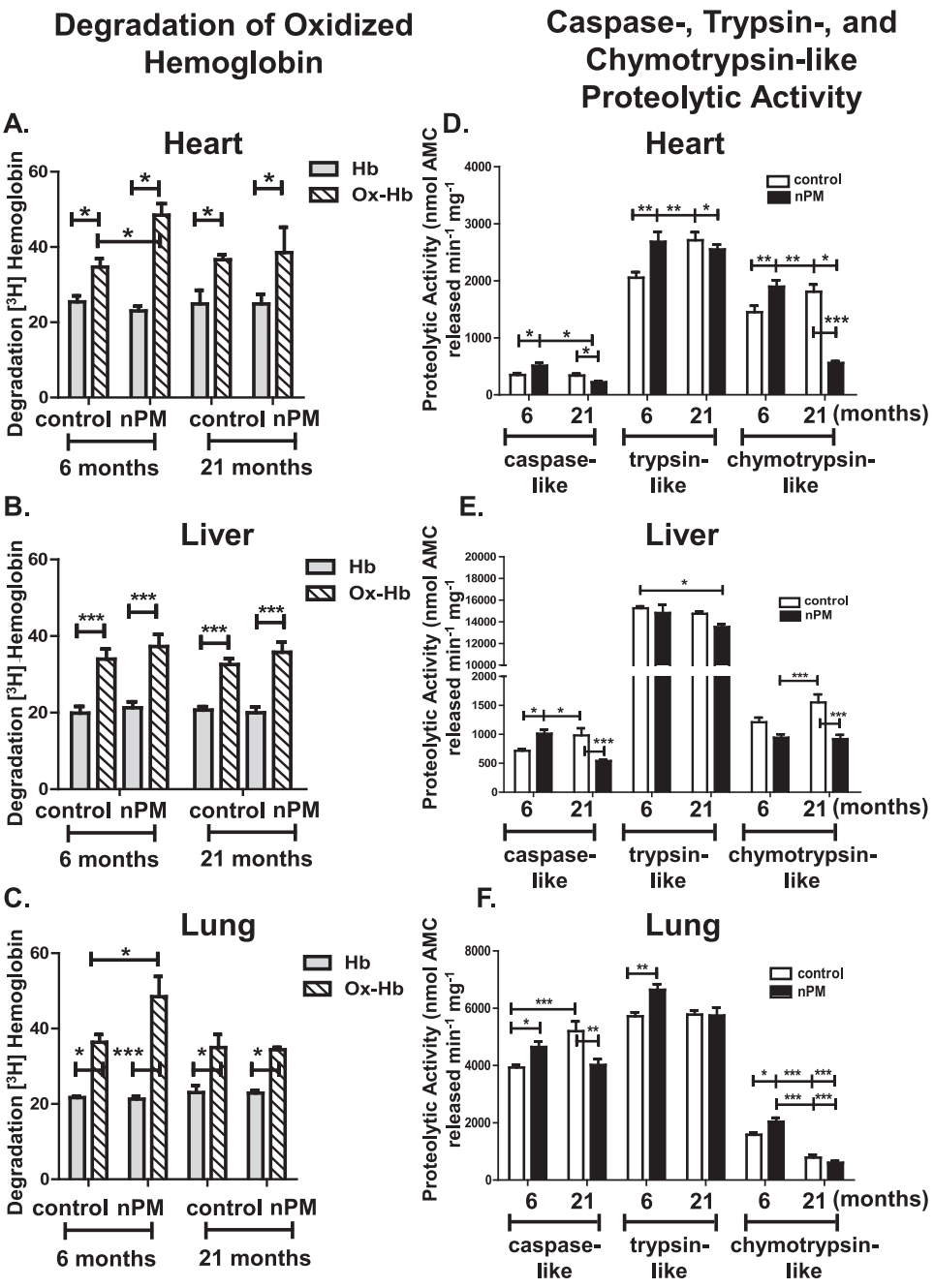

Fig. 2. Preferential degradation of oxidized hemoglobin and caspase-, trypsin-, and chymotrypsin-like activity of the 20S proteasome subunits. 6 month and 21 month old female mice were exposed to filtered air or nPM air prior to tissue collection. (A-C) Tissue lysates were then incubated with either tritium-labeled native-hemoglobin (Hb) (solid) or tritium-labeled oxidized-hemoglobin (ox-Hb) (hashed lines), for measurements of proteolysis. Increased substrate degradation generates higher free scintillation counts corresponding to higher rates of proteolysis. (A) Heart. (B) Liver. (C) Lung. Proteolytic activity of the three catalytic subunits of the 20S proteasome. (D) Heart. (E) Liver. (F) Lung. Error bars denote standard errors of the mean (S.E.M) values. (A-C) Hemoglobin substrate (solid) and oxidized hemoglobin substrate (hashed lines), degradation of hemoglobin versus oxidized hemoglobin substrate within each treatment group was compared and differences in degradation of oxidized hemoglobin, across the four treatment groups, were compared relative to the 6 month control samples. (D-F) Statistically significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicated with asterisk, relative to the 6 month control (6 animals per treatment group) were compared for each fluoropeptide substrate (caspase-like, trypsin-like, and chymotrypsin-like) using one-way ANOVA.

2.2. Preferential degradation of oxidized substrates by the 20S proteasome

Prior studies showed oxidized proteins are the preferred substrate of the 20S Proteasome [65,66]. To demonstrate preference for oxidized substrates, lysate was incubated with either native ([^{3}H]Hb) or oxidized hemoglobin ([^{3}H]Hb$_{oxdized}$), offering a substrate with oxidative modifications specific for Proteasome degradation [65]. Here, basal levels of oxidized hemoglobin (black) were higher in all tissue lysate, compared to native hemoglobin (white). Upon nPM exposure, heart and lung tissue from nPM exposed young females showed increased degradation of oxidized hemoglobin compared to 6 month controls (Fig. 2A,C). Yet tissue from nPM exposed aged females showed no adaptive proteolytic increase (Fig. 2A-C).

2.3. Degradation of 20S Proteasome subunit-selective fluorogenic peptides

The relative proteolytic capacities of the three individual subunits of the 20S catalytic core were measured. Tissues from nPM exposed young females showed at least a 25% higher caspase-like activity in all lysates compared to young controls (Fig. 2D-F). Heart and lung lysates from nPM exposed young mice showed higher trypsin-like and chymotrypsin-like activity compared to age-matched controls (Fig. 2D,F). Aged tissue lysates possessed a basal rise in activity, though this was subunit and tissue specific. Moreover, tissues from nPM-exposed 21 month females showed no difference in proteolytic capacity compared to age-matched controls (Fig. 2D-F).

2.4. Exposure to nPM further increases the presence of oxidized proteins

Increased protein oxidation has been implicated as a hallmark of protein damage [67], and consequently, a measure of aging [68]. Following exploration of the 20S proteasome amount and proteolytic activity in tissue from 6 month and 21 month control or nPM-treated female mice, protein lysate was analyzed for total content of oxidized

proteins. Interestingly, levels of protein oxidation remained relatively unchanged in tissues collected from 6 month controls, 6 month nPM-treated, and 21 month controls (Supporting Fig. 3), which is a similar trend reported in middle-passage cells [69,70] and model organisms [45]. Yet, in all three tissues collected from 21 month females exposed to nPM, caused a marked increase in protein oxidation (Supporting Fig. 3). Indicating that nPM exposure in middle-aged mice may act as an accelerator of protein damage, mirroring increased oxidation evident in chronic age-associated diseases [71].

2.5. Immunoproteasome expression and activity is impacted with age and nPM exposure

The Immunoproteasome is an alternative form of the ATP-independent 20S Proteasome [72]. It was originally characterized for its role in peptide-processing necessary for activation of the adaptive immune response [73]. Since then, our understanding of the Immunoproteasome has evolved, so too has its increasing importance within the cell. Specifically, the Immunoproteasome was shown to be inducible following adaptive pretreatment [74,75] and preferential degradation of oxidized proteins [76]. Moreover, removal of Immunoproteasome subunits in mice led to increased susceptibly to oxidative stress [77]. To address the growing role of the Immunoproteasome in the adaptive stress response, we measured Immunoproteasome-specific levels and activity.

Immunoproteasome-specific LMP2 and LMP7 subunit protein levels were higher in heart and lung tissue from nPM exposed young females (Fig. 3A,C & Supporting Fig. 3A,C). In a similar manner, proteolytic activity of the trypsin- and chymotrypsin-like activity was higher in heart and lung lysate (Fig. 3D,F). In contrast, the liver lysate showed no change in LMP2 or LMP7 levels in 6 month nPM-treated samples (Fig. 3B & Supporting Fig. 3B). Moreover, no change in individual subunit activity was detected between young nPM treated and controls (Fig. 3E). Yet, with age, all tissues showed higher amounts of LMP2 and LMP7 (Fig. 3A-C & Supporting Fig. 3). Interestingly, nPM exposure in 21 month females showed higher amounts of LMP2 and LMP7 in heart lysates (Fig. 3A) and LMP2 in the lung lysate (Fig. 3C). Yet, even with higher amounts, only the 21 month control heart lysate showed levels higher than 6 month controls (Fig. 3D). Neither the liver or lung lysates showed any change (or in some instances, lower activity) in Immunoproteasome trypsin- and chymotrypsin-like activity (Fig. 3E,F). Together, indicating that although there are higher amounts of the subunits, their functional capacity shows no change in an age-dependent manner.

2.6. Expression of the 20S-subunit transcriptional regulator, Nrf2, show an age and nPM-dependent change

Transcriptional activation of stress responsive genes is critical in mediating the transient adaptive response to short term oxidative stress. One of the primary regulators of this process is Nrf2 [78]. Prior work showed low concentrations of hydrogen peroxide caused Nrf2 translocation and binding to electrophile response elements (EpREs), including those of the 20S Proteasome β-subunits [44], ensuring de novo 20S Proteasome synthesis. Here, expression levels of Nrf2 were explored in 6 month and 21 month female mice exposed to either filtered or nPM air.

After nPM exposure in 6 month females, heart and lung tissue lysate show higher amounts of Nrf2 compared to 6 month controls (Fig. 4A,C). Moreover, aging, alone, showed higher Nrf2 levels, in all lysate compared to 6 month controls (Fig. 4A-C). Yet, tissue from 21 month nPM treated mice showed no change in Nrf2 levels compared to 21 month controls (Fig. 4A-C), matching earlier findings shown in aged-matched males (Supporting Table 1) [19]

2.7. Induction of Nrf2-regulated phase II genes, show an age and nPM-dependent increase

A major consequence of impaired Nrf2 transcriptional activation is increased cellular susceptibility to damage accumulation and clearance. Here, we focus on two Nrf2-regulated Phase II detoxification enzymes, heme-oxygenase-1 (HO-1) and the heterodimer components of glutamate cysteine ligase (GCL): the catalytic subunit (GCLC) and the modifier subunit (GCLM), all of which were previously found to be strongly induced in response to exogenous stressors [79,80], including redox-cycling compounds, such as those found in diesel exhaust [81].

After nPM exposure in 6 month females, heart and lung tissue lysate had higher amounts of GCLC and GCLM (Fig. 5A,C) and HO-1 (Supporting Fig. 4A,C) when compared to 6 month controls. Additionally, all tissue lysate showed higher amounts in an age-dependent manner compared to 6 month controls for GCLC, GLCM, and HO-1 (Fig. 5 & Supporting Fig. 4). However, nPM exposure in 21 month females showed no difference compared to age-matched controls (Fig. 5 & Supporting Fig. 4).

2.8. Aging and nPM-exposure, cause higher levels of Nrf2 regulators

Bach1 and c-Myc are negative transcriptional regulators of Nrf2, expressed in a range of tissues [82,83]. Bach1 and c-Myc interact with Nrf2 for binding of ARE/EpRE elements, a role which may be critical in dampening the stress response when no longer necessary. In addition, Keap1 is the cytosolic partner of Nrf2, which under homeostatic conditions, blocks Nrf2 nuclear translocation [21]. Here, we wished to understand the age-related and nPM-impact upon these regulators in 6 month and 21 month female tissue.

Lysates from all tissues (heart, liver, and lung) showed higher levels of Bach1 and c-Myc in 21 month controls compared to 6 month controls (Fig. 6). Interestingly, nPM exposure in 6 month females, showed higher amounts of Bach1 and c-Myc within heart lysate (Fig. 6A), whereas the liver showed only a higher amount of c-Myc (Fig. 6B), and the lung showed no change in either regulator (Fig. 6C). Nor was nPM exposure in 21 month females capable of causing tissue-specific changes compared to 21 month controls (Fig. 6A-C). These findings match prior results in male mice undergoing nPM exposure (Supporting Table 1) [19], indicating the change in Bach1 and c-Myc levels is potentially a compensatory mechanism to turn off the similarly higher Nrf2 levels, evident in aged mice.

In contrast, Keap1 remained unchanged in tissue from young nPM exposed females (Supporting Fig. 5A-C). Nor did it appear to undergo an age-dependent increase. Interestingly, only liver lysate showed higher amounts of Keap1 from young, nPM-treated females (Supporting Fig. 5B). Together, these findings suggest that Nrf2 regulation is not limited by a changing dynamic in Keap1 partner levels, rather a dysregulation in its interaction with other regulators, such as Bach1 and c-Myc.

3. Discussion

Our findings demonstrate that sub-lethal, short-term exposure to vehicular-derived nanoparticles (nPM) results in higher levels of various stress responsive proteins (the 20S Proteasome, Nrf2, GCLC, GCLM, HO-1, the Immunoproteasome, and Lon) in young females. Yet with age, although basal levels of stress responsive enzymes rose, there was no difference in nPM exposed 21 month old females, indicating the homeostatic range, necessary to mitigate cellular damage, is potentially compressed with age, suggestive of an ‘ageing-ceiling’ effect [78].

Importantly, we find that chronic exposure to nPM, representative of vehicular derived air pollution or smog, induces Proteasome and Lon protease expression not only in the lung (which is directly exposed) but also in liver, and heart, organs which are well downstream of the lung. To our knowledge, these are the first deep data on proteostatic

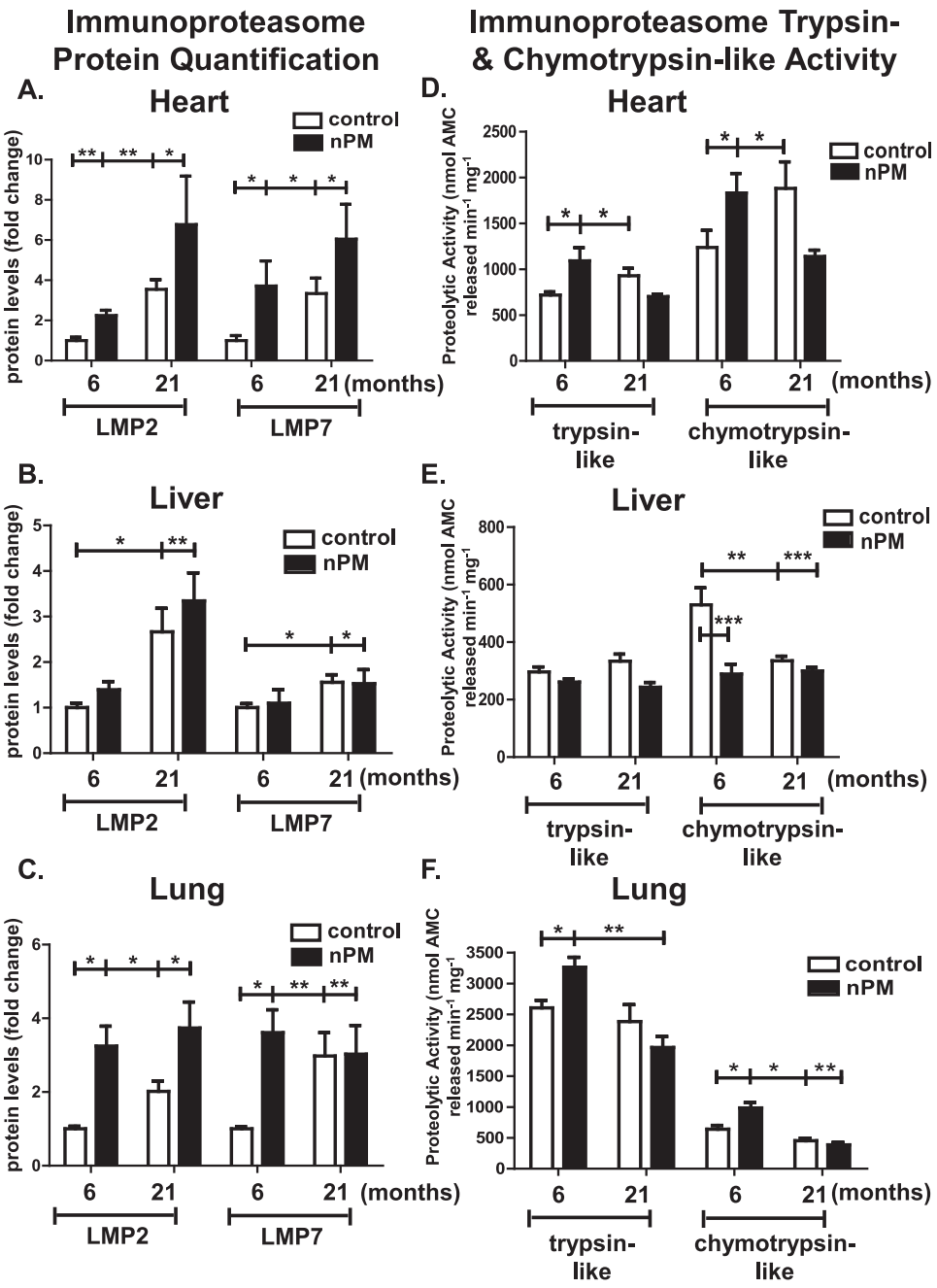

Fig. 3. Expression and activity of the Immunoproteasome changes with age and nPM exposure. Blots are of tissue collected from 6 month and 21 month old females exposed to filtered (white) or nPM air (black). Two of the subunits of the Immunoproteasome were assessed, LMP2 (β1i) and LMP7 (β5i). Levels presented in the bar graph are for the mature form of LMP2 and LMP7. (A) Heart. (B) Liver. (C) Lung. Proteolytic capacity of the Immunoproteasome was measured using fluorogenic peptide substrates specific to Immunoproteasome catalytic subunit activity: (D) Heart. (E) Liver. (F) Lung. Error bars denote standard errors of the mean (S.E.M) values. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicated with asterisk, relative to the 6 month control (6 animals per treatment group).

responses to oxidative damage by air pollution.

These results also support previous findings that demonstrate the activation of the stress response upon nPM exposure is primarily mediated by changing Nrf2 levels, evident in this study, and shown in both rat [20] and mouse models [19]. As well, the higher amounts of Nrf2 was not limited to tissues directly exposed to nPM (i.e. the lung), but extended to indirect tissues (i.e. the heart and liver). This finding follows a similar trend in 6 month and 21 month male mice (Supporting Table 1A,B) [19].

The 20S Proteasome is crucial in preventing the accumulation of protein aggregates. All three tissues showed higher levels in one or all three of the subunits upon nPM exposure in young females. All tissues demonstrated an age-associated rise in the amount of catalytic subunits. Tissue-specific differences in subunit-specific proteolytic activity was evident, with heart and liver lysate showing higher amounts of chymotrypsin-like activity with age, which was the inverse in the lung. The caspase-like activity rose with age in the liver and the lung, but not in the heart. In the presence of oxidized hemoglobin, 6 month nPM treated heart and lung lysate showed higher amounts of proteolysis. These outcomes suggest that although the amount of Proteasome expression (and its transcriptional regulator, Nrf2) were higher with age, the potential induction of proteolytic capacity and proteolysis did not follow suit. A similar trend was originally reported in senescent human BJ fibroblasts, wherein oxidized proteins exceeded proteolysis [84–86]. This suggests one possible explanation: the gradual accumulation of protein aggregates causes cellular perturbation. The cell may try to mitigate damage by increasing Nrf2 levels, causing de novo synthesis of the Proteasome in an effort to clear unwanted aggregates. Unfortunately, these aggregates may exceed the diameter necessary for proteasome degradation. Indeed, studies assessing the components of insoluble cellular aggregates, has shown the 20S Proteasome attaches to these protein aggregates [87]. Thus the 20S Proteasome may try to mitigate cellular damage, but is unsuccessful

As well, other studies suggest a similar trend in the blunting of

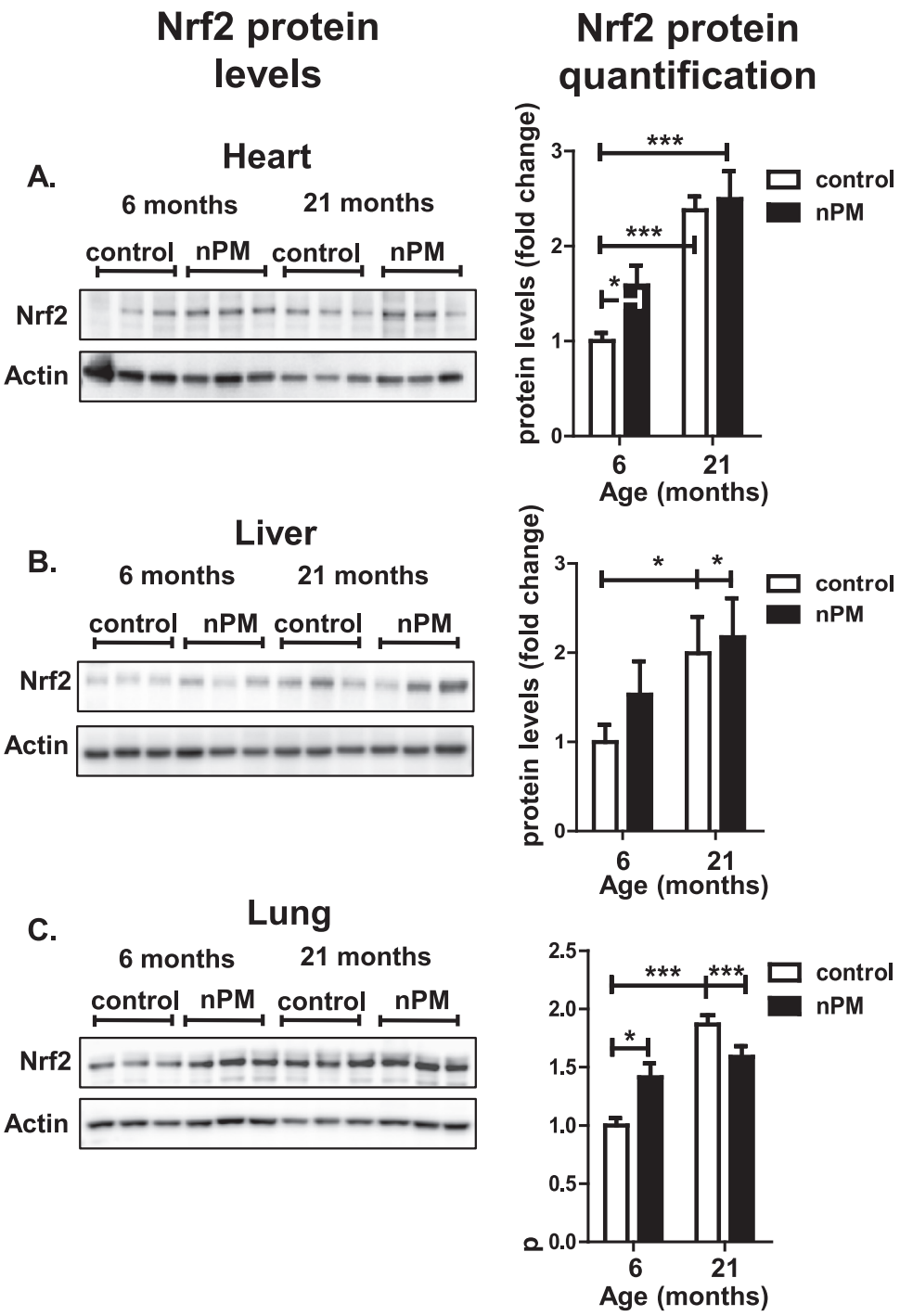

Fig. 4. Expression of the 20S Proteasome Transcriptional Regulator, Nrf2, is nPM- and Age-Dependent. 6 month and 21 month old female mice were exposed to filtered (white) or nPM air (black). Blots were probed with Nrf2-specific antibody and protein loading was normalized to the Anti-Actin-HRP antibody. Images were quantified using ImageJ and presented in the bar graph next to each blot. (A) Heart. (B) Liver. (C) Lung. Error bars denote standard errors of the mean (S.E.M) values. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicated with asterisk, relative to the 6 month control (6 animals per treatment group).

Proteasome activity upon acute exposure to diesel fuel exhaust [88,89]. The lack of protein turnover by the 20S Proteasome, is also evident by the accumulation of protein carbonyls, a marker of protein oxidation [90]. We found significant accumulation of protein carbonyls in 21 month nPM treated tissue, which mirrors similar in vitro particulate exposure [91]. The dichotomy between the age-associated rise in Proteasome levels, but the lack of a concurrent rise in subunit-specific activity, builds upon our findings in the nematode worm [45] and fruitfly [92]. In both cases, an age-related rise in the Proteasome, but inability to adaptively increase amount or activity (and consequently clearance of oxidized proteins). A finding we confirm is conserved in a mammalian model.

In addition, we found the mitochondrial Lon protease levels to rise with age and nPM exposure. As Lon has been previously identified as a stress-inducible protein [93], we confirm this here in a mammalian model. Yet, other studies suggest the converse, with Lon protein levels decreasing with age, shown in both senescent cellular models [69,94] and in aged mice [95]. Arguably, the difference between our findings and those of earlier work is due to age differences. Here, we utilized middle-aged mice, whereas prior studies focused on aged tissue (greater than 24 months), indicating that middle-age is an area that should be studied on its own, and may provide greater insight into the mechanistic transition between young and old.

A unique finding of this study was the impact of nPM-exposure upon the Immunoproteasome. With age, basal activity of the Immunoproteasome was higher in a tissue- and subunit-specific

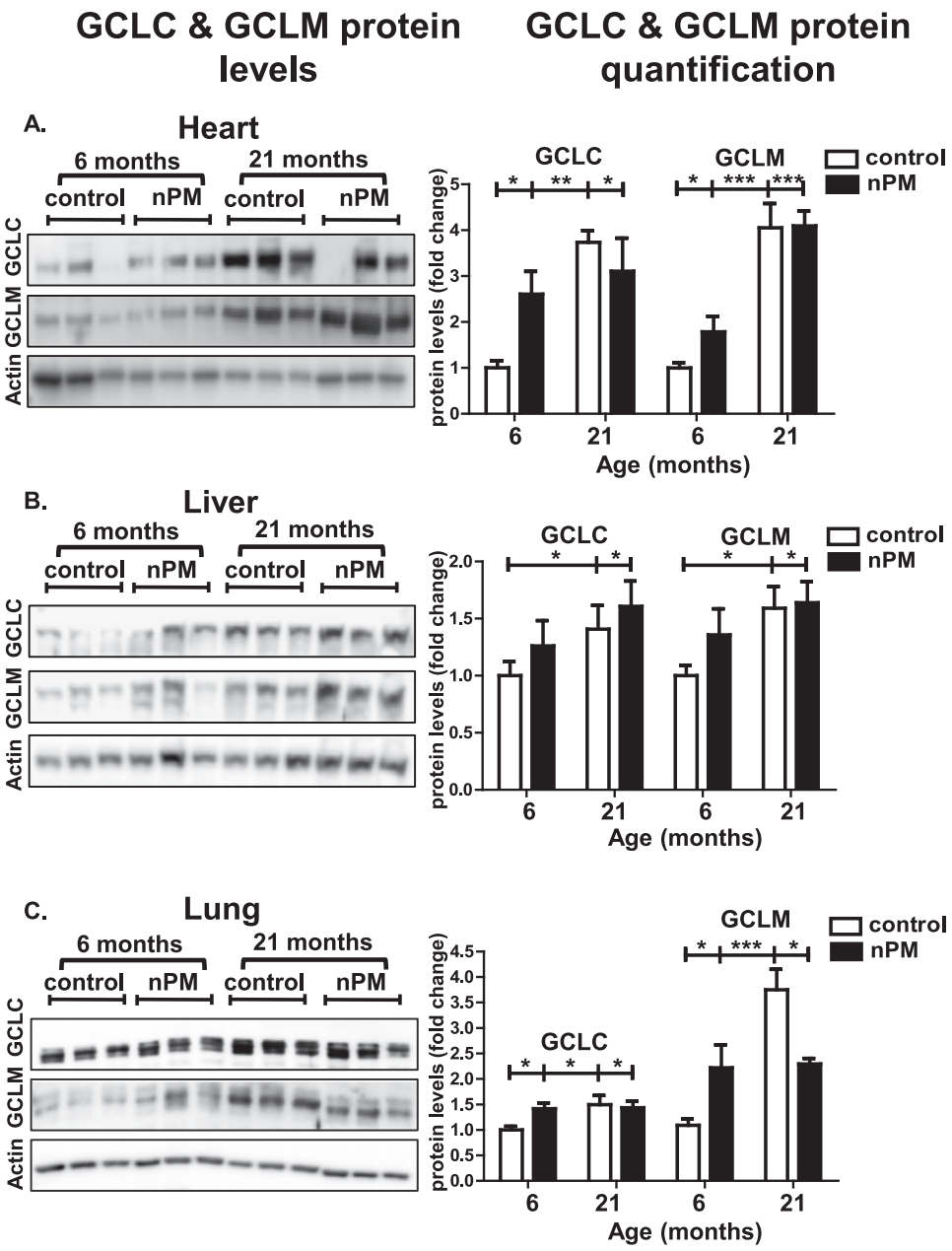

Fig. 5. Induction of Nrf2 Phase II genes, GCLM and GCLC. 6 month and 21 month female old mice exposed to filtered (white) or nPM air (black) were assessed for changes in the glutamate cysteine ligase complex: glutamate-cysteine ligase catalytic subunit (GCLC) and the glutamate-cysteine ligase regulatory subunit (GCLM). Blots were probed with GCLC and GCLM antibodies and samples normalized to the Anti-Actin-HRP antibody. Error bars denote standard errors of the mean (S.E.M) values. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicated with asterisk, relative to the 6 month control (6 animals per treatment group).

manner. Matching prior findings of increased Immunoproteasome in aged rat liver tissue [76]. The change evident in the Immunoproteasome activity (with age-related activity remaining unchanged or declining) is different from that of the 20S Proteasome, and touches on its role in both the pro-inflammatory pathway and oxidative stress response. Originally identified for its role in peptide production, which is necessary for antigen presentation [72], it has since been coined as the 'inducible-Proteasome' [96–98]. This is partly because pretreatment with an adaptive dose of an oxidant was shown to trigger an inducible response. Moreover, in vitro cell culture studies found proteolytic capacity matched that of the 20S Proteasome [13,74]. Indeed, recent studies have indicated a growing role of the Immunoproteasome in various environmental-derived lung diseases [99,100]. Suggesting further work is necessary in exploring the interplay between the 20S Proteasome and the Immunoproteasome.

The Nrf2 regulators, Bach1 and c-Myc, demonstrated an adaptive increase in 6 month nPM-treated females. Bach1, a member of the CNC-related bZIP superfamily of transcriptional factors, possesses a unique BTB domain in its N-terminal region, which has been linked to transcriptional repression [101,102]. Due to Bach1's interaction with stress responsive genes and its high amount of cysteine residues (34 residues), which exceeds the number found on the redox-sensitive Keap1, suggests Bach1 may be a redox-responsive transcriptional mediator, but with the caveat that not all cysteine residues are redox sensitive [103]. Indeed, one study offers evidence for this, as the presence of the sulfhydryl oxidizing agent, diamide, was shown to reverse Bach1 suppression of EpRE target genes [104].

Therefore, as the stress response requires both an activator (Nrf2) and suppressor (potentially Bach1 and/or c-Myc), it is plausible that the interplay between these transcriptional regulators is necessary for an adaptive stress response. Indeed, this temporal relationship is evident when comparing the nuclear translocation of Nrf2 and Bach1 in cell extract treated with cigarette smoke condensate (CSC). Within 6 h of CSC exposure, Nrf2 moves into the nucleus, followed by a delayed exodus (~48 h), coupled with Bach1 nuclear accumulation [105]. Together, suggesting the sequential relationship between Nrf2 and Bach1: the cell is able to combat the immediate stress, via activation of the stress response, yet requires the means to turn-off the response when no

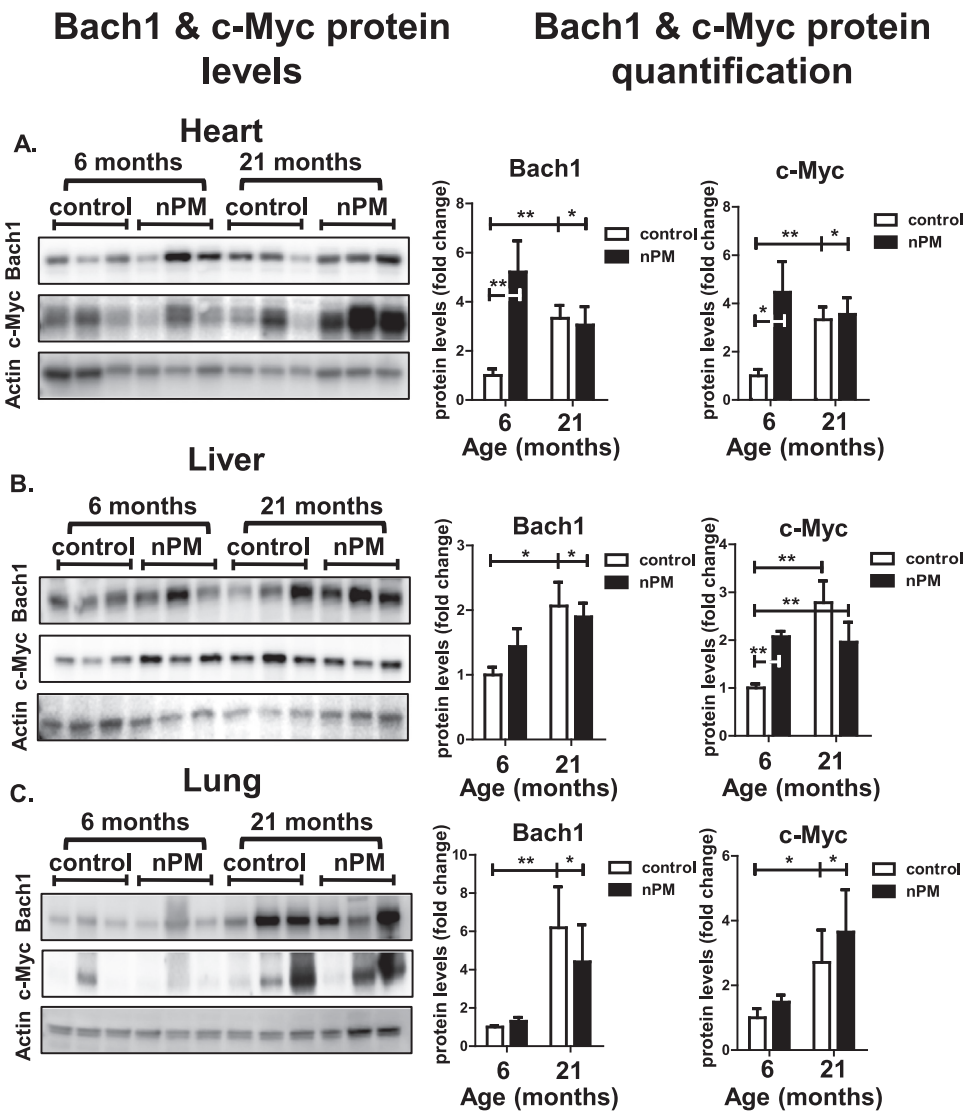

Fig. 6. Expression of the Nrf2 transcriptional competitors, Bach1 and c-Myc, change with age and nPM exposure. Western blot of protein lysate from 6 month and 21 month old females exposed to filtered (white) or nPM air (black). Blots were probed with Bach1 and c-Myc-specific antibodies and normalized to the Anti-Actin-HRP antibody. Images were quantified using ImageJ and levels presented in the bar graph (to the right of each blot). (A) Heart. (B) Lung. (C) Liver. Error bars denote standard errors of the mean (S.E.M) values. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicated with asterisk, relative to the 6 month control (6 animals per treatment group).

longer necessary. Our own findings may suggest a similar occurrence, but further mechanistic studies are necessary.

Interestingly, with age, basal levels of Bach1 and c-Myc rose in all three tissues. A finding that is consistent in prior aging studies [19,106,107]. A similar trend is present in various chronic diseases, most notably, lung-related diseases. In chronic obstructive pulmonary disease (COPD), Bach1 protein amounts rose and exceeded those of Nrf2 [108]. Moreover, age-associated increase in Bach1 and c-Myc limited nPM-mediated adaptive induction, matching findings conducted in male mice (Supporting Table 1A,B) [19]. This suggests that the increased Bach1 expression may act as a compensatory mechanism associated with the age-related increase in inflammation and oxidative stress [109,110].

Although levels of stress responsive enzymes (including the Immunoproteasome, 20 S Proteasome, and the Lon protease) were higher with age, the ability to mitigate an acute oxidative insult by transient adaptation was compromised in older animals. Evident when comparing the potential inducibility of these enzymes in young nPM treated females compared to middle-aged nPM treated females: young show an increase in expression, yet with age, as basal levels have potentially already reached a maximal threshold, or an 'aging-ceiling effect', induction is no longer possible.

In addition, exploration of the impact of nanoparticulate exposure ('traffic-derived smog) upon activation of the stress-protective pathways in female mice has not been previously explored. This is important as evidence indicates sex-specific differences in lifespan [111] and healthspan [112,113]. However, exploration of sex disparities in health-promoting interventions have only recently garnered attention, so much so that the United States National Institutes of Health has mandated consideration of sex as a biological variable [114,115]. Our findings suggest significant similarities in males that underwent the same treatment (Supporting Table 1A,B) [19]. A new aspect of the present study was the inclusion of measures of proteostasis, and adaptive responses of proteolytic enzymes. Thus, although the present study indicates similar declines in many aspects of adaptive homeostatic responses with age in both males and females, we now need to explore new studies of proteostasis and proteolytic adaptation with males. This is especially important as we try to determine the reason(s) for female-specific responses to chronic nanoparticulate matter ('smog') that are implicated in increased risk for neurological impairment and disease development [6].

Overall, these findings suggest additional factors that need to be assessed in understanding the transition from young to old. Specifically, the interplay between Nrf2 and its potential inhibitors, such as Bach1 and c-Myc. This is important because, with age, the adaptive homeostatic set-point appears to shift. In our comparison of young and middle-aged tissues, we found that basal levels increased, yet induction, clearly evident in young, does not occur with age. A trend that matches our

findings in model organisms. In turn, this suggests that with age, the homeostatic set-point shifts upward, limiting any future induction. This potentially results in animals that are more susceptible to damage, and are at a greater risk for mortality.

4. Experimental procedures

4.1. Animal exposure to nanoparticles

C57BL/6NJ female mice (3 months and 18 months) were kept under standard conditions with ad lib food and water access. Particles were collected and prepared as previously described [116]. Briefly, reaerosolized particles (nPM air) or particle-free filtered air (control air) were delivered to sealed exposure chambers for 5 h/day, 3 days/week for 10 weeks, with composition of reaersolized nPM as previously described [116]. Animals did not have evidence of respiratory distress, nor did they lose weight, indicating deliverance of sub-lethal dosage. Following the 10 weeks, animals (6 months and 21 months) were euthanized after isoflurane anesthesia, tissue was collected and stored at − 80 °C. Animals were in four treatment groups (6 months control, 6 months nPM, 21 months control, and 21 months nPM), with 6 animals per treatment group. All procedures were approved by the USC Institutional Animal Care and Use Committee, and animals were maintained under standard conditions according to the NIH guidelines.

4.2. Preparation of mouse samples

Each treatment group consisted of 6 animals, providing 6 biological replicates per tissue analyzed (heart, liver, and lung). From each biological replicate, 5 mg of tissue was transferred to 2 mL reinforced tubes containing 2.8 mm ceramic beads (no. 19–628, Omni International) containing 500 μL mPER buffer (no.19–040, Thermo-Fisher Scientific,), absent protease inhibitors. Samples underwent 3 cycles of 15 s on/30 s off in the Bead Ruptor 24© (no. 19–040, Omni International), before lysate was transferred to fresh tubes. Protein concentration was measured using the bicinchoninic acid (BCA) assay, with reducing agent (no.23252, Pierce).

4.3. Western blot

Protein samples were run on 10%, SDS-PAGE gels (no. 4561036, Bio-Rad), and transferred to a PVDF membrane (no. 1620177XTU, Bio-Rad). Antibodies used for protein detection are in Supporting Table 2. Primary antibodies were incubated, overnight at 4 °C (no. 37543, Thermo-Scientific). Blots were washed for 3 × 10 min in washing buffer: 100 mL TBS 10 × (no. 46-012-CM, Corning), 500 μL Tween (no. P1379-500 mL, Sigma-Aldrich), and total volume was raised to 1000 mL with deionized water. Blots were incubated in secondary antibody for 1 h at room temperature, at which point the washing step was repeated an additional time. Commercially-available chemiluminescent kit (no. 32132, Thermo-Fisher) was used for detection of protein bands by chemiluminescence. Afterwards, blots were stripped by incubating in 15 mL commercially-available stripping buffer at room temperature (no. 21059, Thermo-Fisher). Blots were then washed in washing buffer as above.

4.4. Fluoropeptide proteolytic activity assays

Proteolytic activity of 5 μg of lysate was measured in triplicate in a 96-well flat-bottom black plate, suspended in proteolysis buffer (50 mM Tris, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, pH 7.5), with 2 μM of Proteasome subunit-specific substrates: Caspase-like/β_1 activity, Z-LLG-AMC (no. 539141, Calbiochem), Trypsin-like/β_2 activity, Z-ARR-AMC (no. 539149, Calbiochem) Chymotrypsin-like/β_5 activity, Suc-LLVY-AMC (no. 539142, Calbiochem). Immunoproteasome subunit-specific substrates were added: Tryspin-like/β_{1i} activity, Ac-PAL-AMC (no. S-310, BostonBiochem) and chymotrypsin-like/ β_{5i} and β_{2i} activity, Ac-ANW-AMC (no. S-320, BostonBiochem).

Fluorescence readings were recorded every 10 min for 4 h using an excitation wavelength of 355 nm and an emission wavelength of 444 nm. Fluorescence units were converted to moles of free 7-amino-4-methylcoumarin (AMC), using an AMC standard curve of known amounts (no. 164545, Merck), with background subtracted.

4.5. Preparation of [^{3}H]-labeled substrates

Tritium-tagged oxidized-hemoglobin ([^{3}H]-OxHb) was generated as previously described [66]. Briefly, 5 mg of hemoglobin was dissolved in 0.1 M Hepes buffer with the addition of 6.6uCi [H3]Formaldehyde and 20 mM sodium cyanoborohydride [117]. Mixture was incubated at room temperature on an end-over-end shaker for 1 h. At which point, hydrogen peroxide (H_2O_2) was added at a final concentration of 5 mM, and the mixture was rocked for an additional hour. Mixture was dialyzed through a 10,000 MWCO filter (Millipore) at 15,000 g for 30 min, eluent was discarded, and slurry re-suspended in Hepes buffer. This was repeated for an additional 7 washes to remove unbound [^{3}H]Formaldehyde. Protein content was quantified using BCA assay kit (no.23252, Pierce).

4.6. [^{3}H]-labeled substrates proteolytic activity assay

5 μg of oxidized [^{3}H] hemoglobin (([^{3}H]-OxHb) was added to 15 μg of cell lysate. Samples were incubated on a plate shaker at 300 rpm for 2 h at 37 °C. To precipitate any remaining intact protein, 20% trichloroacetic acid and 2% BSA was added. Samples were centrifuged at 13,000 rpm for 10 min and supernatant was collected and added to scintillation fluid. The release of acid-soluble counts were read on a scintillation counter. Background was subtracted and the amount of liberated radiolabel was reported.

4.7. Carbonyl content assay

The protein oxidation detection kit, Oxyblot (no. S7150, Millipore) was used to perform immunoblot detection of oxidatively modified proteins. 5 μg of protein from tissue (6 animals per group) was prepared according to the manufacture instructions. Carbonyl groups in samples were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). Samples were run on a 10% SDS-PAGE gel and transferred to a PVDF membrane for western blot analysis as described previously. Immediately following transfer, blots were reactivated by quickly submerging in ethanol to reactivate the membrane before incubating for approximately 30 min, at room temperature, in ponceau stain (no. 51284, VWR) to measure total protein loading. To remove background stain, blots were washed in 5% acetic acid (25 mL 100% acetic acid and 500 mL deionized water) until bands were visible and scanned in. To completely remove Ponceau stain, blots were incubated at room temperature in 9 mL washing buffer (as described above) and 1 mL 1 M NaOH, until stain was completely removed. To detect carbonylated proteins, blots were incubated, overnight, with the mouse monoclonal anti-DNP antibody, according to manufacturer's instructions (no. MAB2223, Millipore).

4.8. Statistical analysis

6 biological replicates were used for each treatment group, resulting in 24 animals used in the study. Data is expressed as mean ± standard error. GraphPad Prism 6.0 was used for statistical analysis, with statistical significance indicated with the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The one-way ANOVA and Tukey test were used in comparison for protein levels and proteolytic activity.

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Author contributions

LCDP and KJAD designed experiments which were conducted by LCDP, and by MC who performed Western blots and activity assays. NW, TEM, & CEF designed and executed mouse treatment experiments. PP & CS collected nPM and re-aerosolized for exposure. LCDP & KJAD conducted experimental analysis. LCDP, KJAD, MC, NW, TEM, CEF, and HJF all contributed to manuscript preparation (both text and figures) and several rounds of revisions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2018.04.574.

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