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In Old Mice, Exercise Induces Inflammation and Fibrosis Unless Alk5-Inhibitor and Oxytocin Are Used

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ABSTRACT

Exercise and diet are the best-known methods for attenuating aging-related health decline. However, exercise in older age has diminished gains of strength and agility, and a danger of unrepaired muscle damage. Improving the understanding of age-related differences in response to exercise, our results demonstrate that in old mice, downhill treadmill (eccentric) exercise causes increased influx of CD45+ cells (inflammation) and fibrotic index (fibrosis) in the heart and skeletal muscles. To explain these changes, we identified newly synthesized proteins through bio-orthogonal noncanonical amino acid tagging (BONCAT) and established that exercise exacerbated age-associated protein patterns through a dysregulated transforming growth factor (TGF)-β, Ras/MAPK/PI3Akt, and JAK/STAT pathways. Testing causality, we found that an inhibitor of TGF-β (Alk5 inhibitor, A5i) in combination with the age-diminished peptide oxytocin, previously shown to rejuvenate muscle and brain in sedentary animals, allowed aged mice to exercise without pathologies of skeletal and heart muscles and youthfully restored their de novo proteomes.

1 | Introduction

Aging is characterized by chronic inflammation, increased tissue fibrosis, skewed immune responses, accumulated tissue damage, and diminished tissue functionality (Guo et al. 2022; Poulose and Raju 2014; Owen-Woods 2020). These changes lead to physiological decline across all organ systems, lowered physical endurance and functional fitness, lowered aerobic capacity, and weakened cardiovascular endurance, especially after the age of 60 (Tomás et al. 2018; Wickramarachchi et al. 2023).

One example is the exercise-caused damage to skeletal muscle, also referred as exercise-induced muscle damage (EIMD). In young mice and people, EIMD is typically followed by a transient healthy inflammatory response that overlaps and positively contributes to functional regeneration and repair (Tidball et al. 2021; Kunz and Lanza 2023). However, the gains of exercise, such as increased strength and agility, decline with aging, while inflammation and tissue damage persists at the expense of repair (Grevendonk et al. 2021; Peake et al. 2010; Li et al. 2024a). This likely contributes to the lack of motivation to exercise in older populations, even though, exercise is the best way to prevent sarcopenia or muscle loss, and to improve the overall health, via its stimulation of tissue regeneration (Saito et al. 2020; Liu et al. 2023), angiogenesis (Chen et al. 2022), insulin sensitivity (Holloszy 2005), and brain plasticity (Nichol et al. 2009). EIMD can be linked to unaccustomed exercise load, duration, magnitude, and intensity, with the degree of damage persistence being highly influenced by an individual's age, sex, and comorbidities. Amid the growing evidence of EIMD, its impact on overall health is not well characterized, especially its acute effects among elderly, who have higher risk of getting exercise-related injuries (Little et al. 2013).

Our results demonstrate that downhill treadmill exercise, even when tuned down to accommodate the aged animals, rapidly promotes fibrosis and inflammation in skeletal muscle and heart in old mice and alters the profile of de novo synthesized tissue proteomes toward an inflammaging. These detriments were not observed in young-exercised animals. Considering the many positive pleiotropic effects of exercise, we did not seek to replace all of these with a single drug, but rather focused on a way to diminish exercise-caused damage in the old, restoring their ability to benefit from this health-extending activity. To understand the mechanism of the observed changes, we used bio-orthogonal noncanonical amino-acid metabolic proteomics (BONCAT) to label de novo synthesized proteins. BONCAT identifies de novo synthesized proteins at a specific experimental time, for example, in response to exercise, via the in vivo tagging with azido homo-alanine. We identified TGF-β as the main signaling hub that becomes skewed by exercise in the muscle and heart tissues of old mice.

To test causality, we administered an Alk5 inhibitor (A5i) and age-diminished peptide-hormone oxytocin (OT) to old, exercised mice. We used the combination because compared to A5i alone or OT alone, their combination allows lowering the dose of A5i, which is important for preventing many side-effects of overtly diminished TGF-β signaling, and broadens the rejuvenation effects in sedentary old mice (Elabd et al. 2014; Mehdipour et al. 2019; Yousef et al. 2015). Together, this treatment completely reversed the exercise-caused detriments in old mice, prevented inflammation and fibrosis of skeletal and cardiac muscles, and restored hundreds of signaling proteins to their more youthful levels.

2 | Results

2.1 | Exercise Induces Inflammation and Fibrosis in Old Skeletal Muscle and Heart

As exercise is one of the most common activities in all age groups known to induce muscle injury (Li et al. 2024a; Köhne et al. 2016; Lazarus et al. 2019), and old mammals do not repair muscle injury well, we decided to determine the impact of exercise on muscle and heart of old mice. We subjected old (22–24 months) and young (2–4 months) MetRS^{L274G} male and female mice to accustomed voluntary downhill treadmill running at a −14-degree slope for 60 min. We selected an exercise regimen that was well tolerated by the old animals, even though young mice were capable of more strenuous routines. We assessed the levels of inflammation and fibrosis in the gastrocnemius (GA) hind leg skeletal muscles and in the heart. In parallel, we studied the same parameters of fibrosis and inflammation in muscle and heart of the mice which had cardiotoxin (CTX)-induced experimental muscle injury to their GA, as this is the established model for muscle injury known to induce transient and reproducible acute muscle injury without affecting the nerves and vasculature (Mehdipour et al. 2019; Wang et al. 2022). The schematics of these studies are shown in Figures 1A and S1A.

GA muscles and whole hearts were cryosectioned to 10-micron at 7 days post-injury, using uninjured, non-exercised animals as controls. Immunofluorescence was performed for the CD45 pan-leukocyte marker to detect inflammation, and trichrome histological staining was done to quantify the degree of fibrosis.

The data demonstrated that without exercise or CTX injury, there were no significant or age-different numbers of CD45+ cells in GA, while cardiac inflammation was generally elevated in the old, non-exercised mice, as compared to young (Figures 1B–E and S1B–E). The downhill treadmill exercise and CTX injury both resulted in higher numbers of inflammatory CD45+ cells in the skeletal muscle (Figures 1B,C and S1B,C) and cardiac tissue (Figure 1D,E) of old mice compared to young. Of note, the exercise-related increase in CD45+ cells was more prominent in males, as compared to females (Figure S2A,B).

Fibrosis was not significantly different in muscle and heart tissues of sedentary, uninjured old mice, as compared to young. However, in the exercise groups, there was a clear age-specific difference. Namely, fibrosis was not increased after exercise in the young animals, while the old mice exhibited significant fibrosis of skeletal muscle and heart tissues (Figure 2A–D). Of note, old skeletal muscle had increased interstitial fibrosis in agreement with previous observations on the effects of forced and highly repetitive movements (Barbe et al. 2020).

The old heart tissue exhibited perivascular fibrosis, characterized by increased amount of collagen around the blood vessels, which is often associated with perivascular inflammation, oxidative stress, and activation of fibroblasts and endothelial cells (Ytrehus et al. 2018). In the old mice, the degree of exercised-caused muscle and heart fibrosis was similar to that after CTX injury (Figure S3A–D). In contrast to the higher inflammation in male muscle and heart (as compared to the female tissues), no significant sex-related differences were observed for fibrosis (Figure S2C,D).

These result establish that fibrosis and inflammation are rapidly and significantly increased in the skeletal muscle and in the heart of old mice after exercise, to a degree which is comparable to that caused by muscle injury with cardiotoxin. Young mice can exercise without such fibrosis or inflammation.

2.2 | Exercise Exacerbates Molecular Signatures of Inflammaging in the Old Mice

Next, we addressed the molecular mechanisms of the negative effects of exercise in the old animals. To identify the changes specifically in response to exercise, we performed the bio-orthogonal metabolic proteomics, BONCAT, which identifies de novo synthesized proteins, using MetRS^{L274G} transgenic young and old mice (Liu et al. 2017).

MetRS^{L274G} mice were administered with azido-nor-leucin (ANL) for 7 days, daily, during their treadmill exercise, after which, their skeletal muscles and hearts were assayed using RayBiotech BONCAT antibody arrays as in (Liu et al. 2017, 2022) to quantitatively and specifically detect the exercise-elicited proteome changes (Figure 3A).

This identified the proteins that were produced during and after the period of exercise and uncovered significant differences for

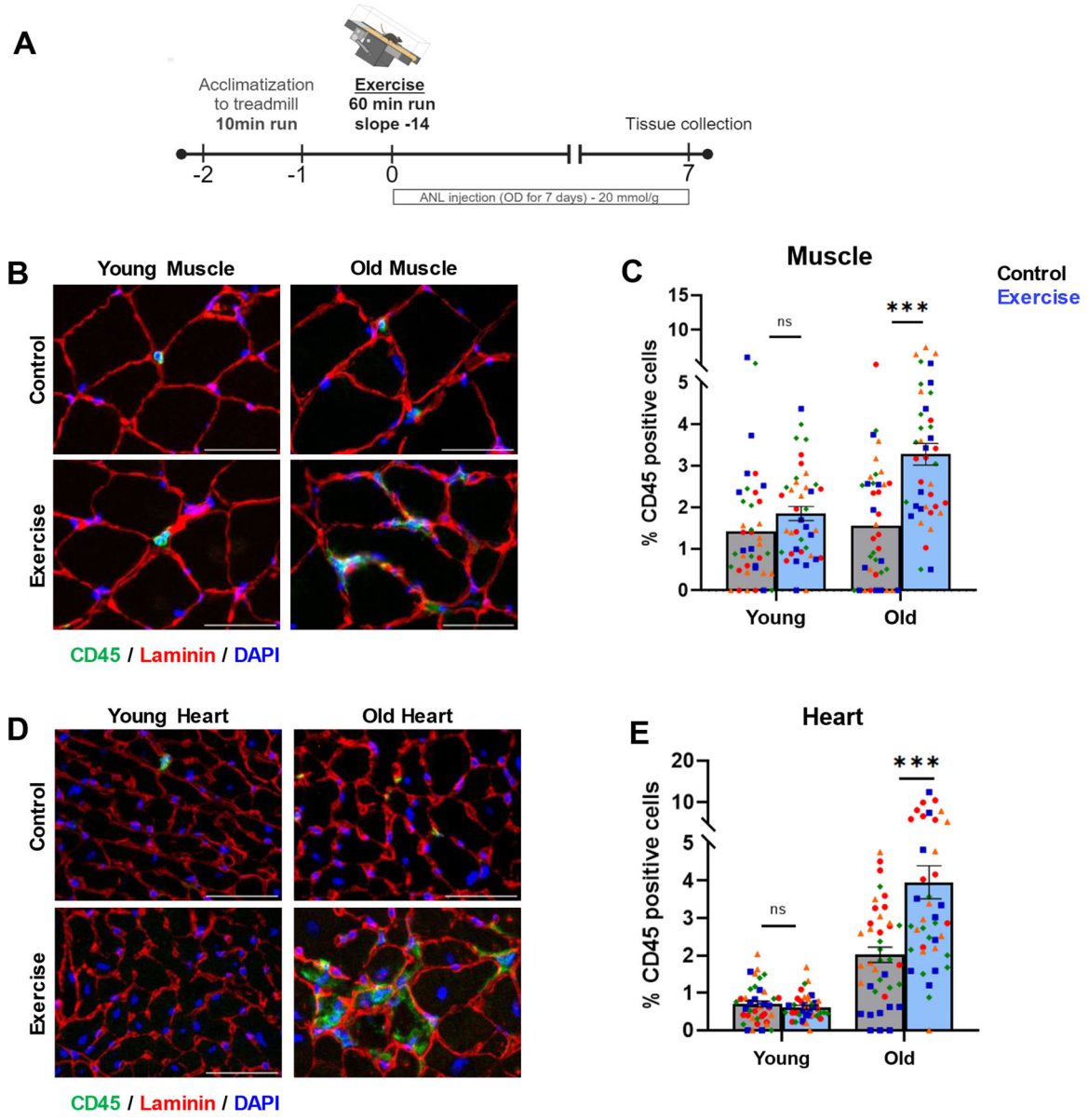

FIGURE 1 | Inflammation is elevated in old muscle (gastrocnemius, GA) and heart tissues after exercise. (A) Schematic of the experimental procedure. Young (2-4 month) and old (22–24 months) male and female MetRS^{L274G} mice underwent exercise on a downhill treadmill at a slope of −14 degree. Representative immunofluorescent images for macrophages using CD45 in (B) muscle and (D) heart of young and old mice after injury. Shown are CD45 (green), Laminin (red), and DAPI (blue). Quantification of the number of CD45-positive cells in (C) muscle and (E) heart after exercise. The data are presented as percentage positive cells in all the micrographs acquired for each mouse. A different color and shape identify individual mice within a group. $N = 4$. Scale bars = 50 μm. Data are mean ± SEM, $^{***}p \leq 0.001$, ns = not significant.

each tissue and age group. Uniform Manifold Approximation and Projection (UMAP) demonstrated separation of the old, exercised (OE) group from other groups: young control (YC), young, exercised (YE), and old control (OC) in both GA and the heart (Figure 3B). Very interestingly, a significant over-production of the newly synthesized proteins was detected by the array in response to exercise in old mice, as compared to young (Figure S4A–D).

To better understand these differences, we compared differentially expressed proteins (DEPs) ($p < 0.05$, fold change > 2) between the OE and OC groups. These analyses identified 86 DEPs in GA, of which 84 were upregulated with exercise, and 2 were down-regulated; and 20 DEPs in heart, of which 11 were upregulated with exercise and 9 were downregulated (Table S1). Figure 3C shows the overlaps among the DEPs. OE heart and GA shared the same four upregulated inflammatory proteins, as compared to the OC: tissue inhibitor of metalloproteinases 2 (TIMP-2), ubiquitin, macrophage-derived chemokine (MDC), and Tie-2, indicating accelerated tissue degradation and inflammation. No down-regulated proteins were similar across the tissues.

To characterize the functions of the DEPs, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway bioinformatics analysis among the DEPs (Figures 3D,E and S4E). With respect to biological processes (BPs), the upregulated in the old DEPs in GA and heart were enriched for cytokine-mediated signaling pathways, leukocyte

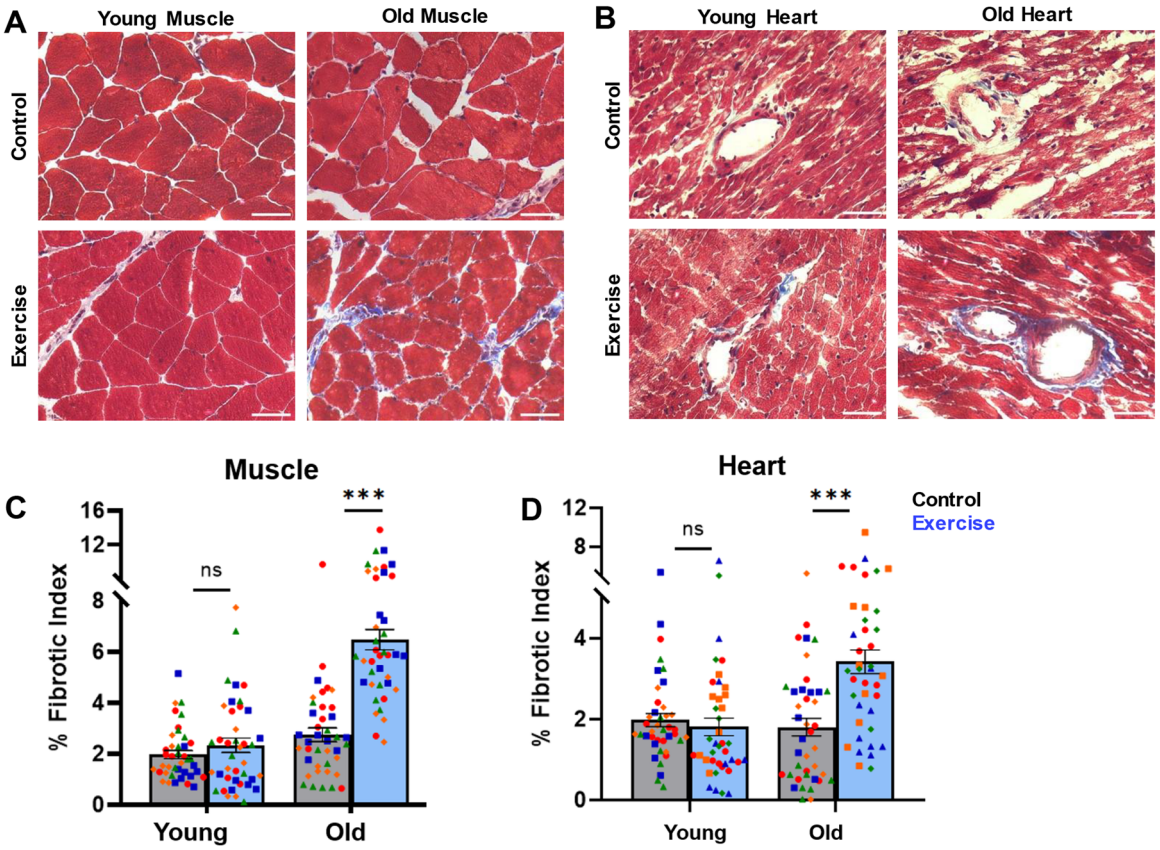

FIGURE 2 | Cardiac fibrosis is aggravated in old mice after muscle injury. (A), (B) Representative trichrome images of muscle (gastrocnemius, GA) and the heart in young and old mice after exercise. (C, D) Fibrotic index of GA and heart after exercise, respectively. The data are presented as a percentage of fibrotic area over total tissue area in all micrographs acquired for each mouse. A different color and shape identify individual mouse within a group. $N = 4$. Scale bars = 50 μm. Data are mean ± SEM, $^{***}p \leq 0.001$, ns = not significant.

proliferation, migration, chemotaxis, and JAK-STAT, in agreement with the exercise-induced inflammation of these tissues.

On the other hand, for the YE group, we found 19 DEPs in GA, all of which were downregulated, as compared to the YC (Figure S4A). These DEPs mainly contribute to chemotaxis, leukocyte migration, and chemokine signaling, suggesting anti-inflammatory effects of the exercise in the young animals (Figure S4E–G).

In the young heart, there were 26 DEPs (25 upregulated and 1 downregulated) (Figure S4C). The upregulated proteins in the heart were enriched in vascular development and angiogenesis in GO and JAK-STAT and pathway in KEGG, as needed for healthy tissue maintenance and repair (Figure S4E–G) (Kim et al. 2022). To analyze the pathway interactions that are influenced by the exercise in the old mice, we constructed a protein–protein interaction (PPI) network of the DEPs (OE v OC) using STRINGdb (Figure 3F). Using the Markov clustering algorithm, we identified the top protein clusters per tissue. Supporting the GO and KEGG, these analyses established that in muscle and heart of old mice, a network of proteins that participates in the TGF-β pathway is identified as a nodal hub of response to exercise.

In recent reports, platelet factor 4 (PF-4) and glucagon-like peptide-1 (GLP-1) were suggested to be the age-diminished, exercise-elicited positive factors (Pham et al. 2019; Wu et al. 2022; Izquierdo 2024), while interleukin 11, IL-11, was proposed as the dominant negative age-elevated protein (Widjaja et al. 2024). Examining the levels of these proteins in our experimental set-up, we did not observe age-specific, or exercise-elicited differences in PF-4 and GLP-1; however, a significant age-associated increase and exercise-elicited decrease were detected for IL-11 (Figure S5).

Summarily, the changes in de novo-produced systemic proteomes demonstrate that signatures of aging are exacerbated in the old mice by exercise, with a dominant signature of activated TGF-β signaling and its cross-talks. The results of BONCAT proteomics strongly support the conclusions from the CD45+ immunofluorescence and trichrome data, that is, in old animals, there are elevated inflammation and fibrosis in heart and skeletal muscles after exercise. The healthy, productive signaling proteins for tissue remodeling and repair were confirmed by this proteomics approach in the young animals.

2.3 | Alk5i + OT Alleviates Exercise-Induced Inflammation and Fibrosis and Calibrates the De Novo Proteome to Its Youthful Profile

After finding that the TGF-β signaling pathway was strongly exercise-upregulated in the old muscle and heart, and considering that age-related increase in TGF-β is associated with elevated fibrosis and inflammation (Ren et al. 2023), we tested if an Alk5 inhibitor (A5i) of the TGF-β receptor would alleviate

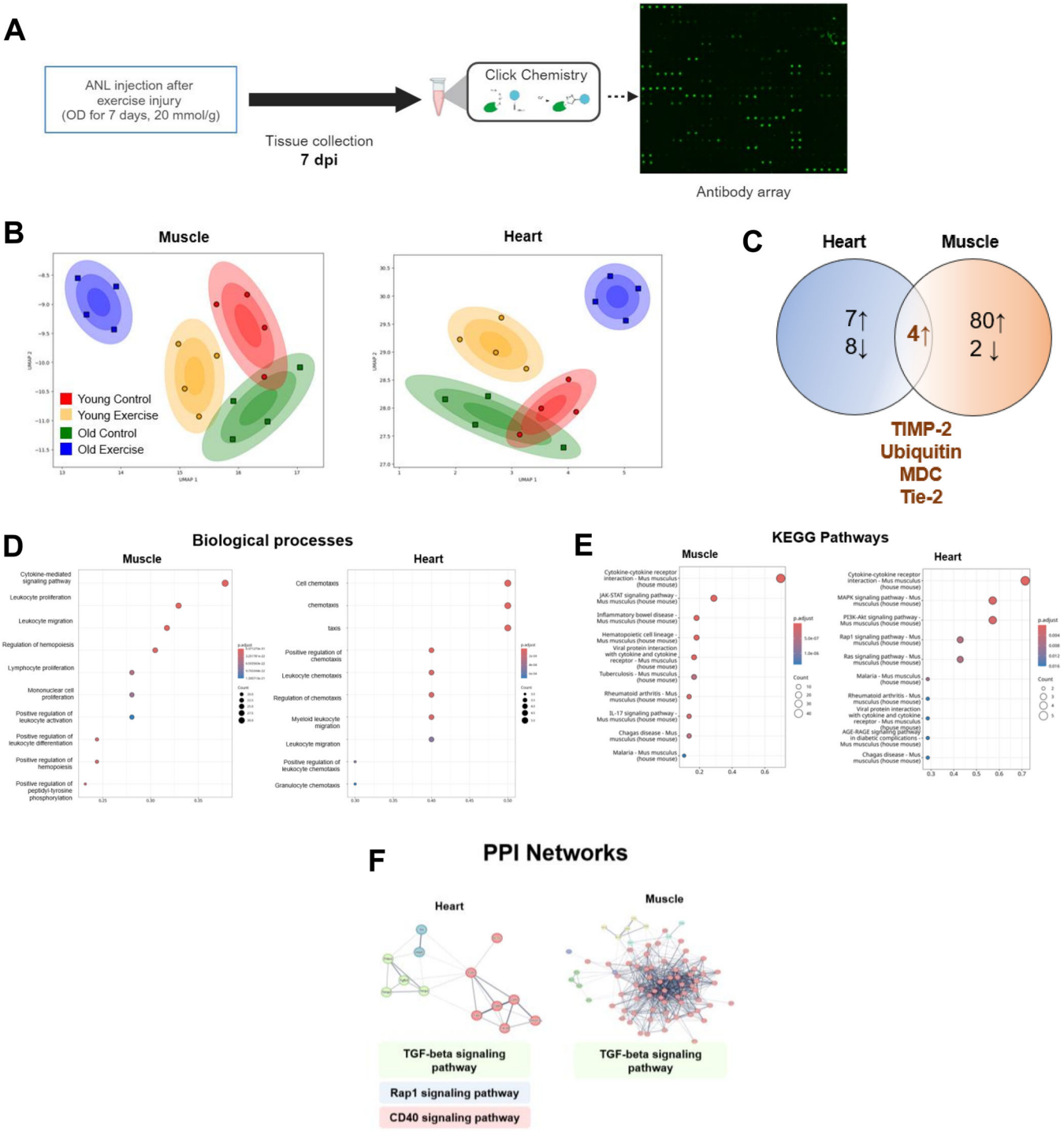

FIGURE 3 | Quantitative proteomic analysis reveals broad systemic inflammatory changes after exercise. (A) Representative images of scanned antibody arrays performed with muscle (gastrocnemius, GA) and heart from young and old MetRS^{L274G} collected 7 days postexercise. (B) Uniform Manifold Approximation and Projection (UMAP) plots of the protein expression profiles of young control (YC), young exercise (YE), old control (OC), and old, exercised (OE) groups in muscle and heart. (C) Venn diagram representation showing the significant differential protein expression in OE versus OC group of muscle and heart tissue. (D, E) Network dot plots showing the enriched GO and KEGG terms of upregulated differentially expressed protein (DEP) in muscle and heart of old mice after exercise. No upregulated DEP was found in TA. (F) STRING network plots showing the protein–protein interaction (PPI) of DEPs heart and muscle clustered using the Markov clustering algorithm (with an inflation parameter = 3).

the negative effects of exercise in old animals. Additionally, we decided to use exogenous oxytocin, known to have positive effects on heart, immune system, and wound healing (Elabd et al. 2014; Wasserman et al. 2022; Sorg et al. 2017) in combination with A5i. As compared to each drug alone (Elabd et al. 2014; Yousef et al. 2015), A5iOT has broader positive effects, reversing multiple phenotypes of aging, e.g., neuroinflammation, liver and muscle fibrosis, and improving the maintenance and repair of the old tissues (Mehdipour et al. 2019). The addition of oxytocin also allows lowering the

dose of A5i by 10-fold, which is important, as overt inhibition of TGF-β signaling has numerous deleterious side-effects, for example, skewed immune response, diminished angiogenesis, and perturbed differentiation of stem and progenitor cells (Mehdipour et al. 2019; Deng et al. 2024).

Animals were administered with A5iOT at 0.02 nmol/g-day and 1 μg/g-day, respectively, or HBSS as vehicle control, for 7 days of their daily exercise regimen, as published in (Mehdipour et al. 2019) (Figure 4A). Skeletal muscles and hearts were cryosectioned, the numbers of CD45+ inflammatory cells and

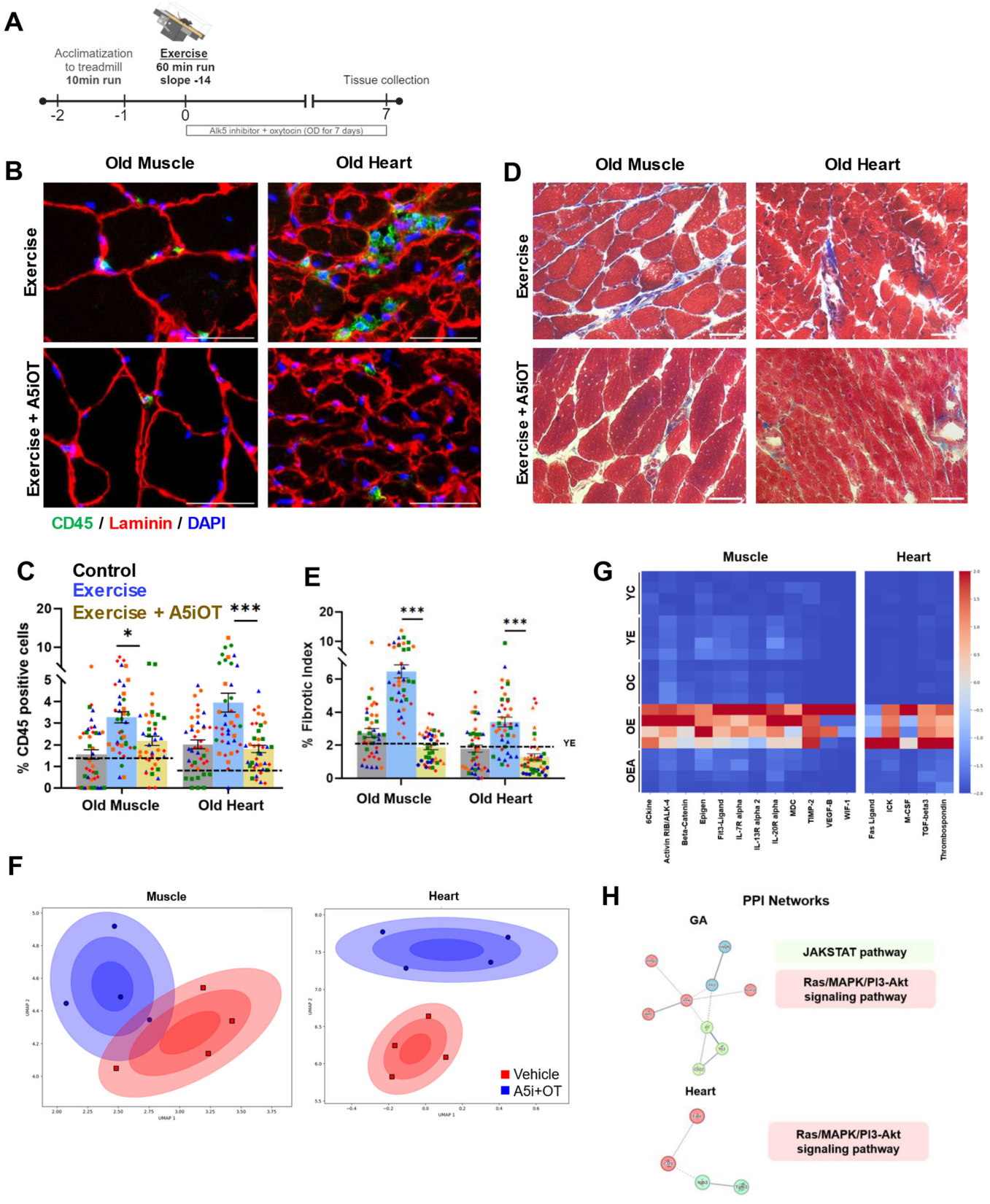

FIGURE 4 | Legend on next page.

the degree of fibrosis were quantified by immunofluorescence and trichrome staining, respectively, as described above.

A5iOT treatment significantly decreased the numbers of CD45+ cells in the muscles of old, exercised mice, making them statistically similar to those found in young mice or in the non-exercised mice (Figures 1B–E and 4B,C). The same positive outcome was seen in the heart, where the exercise-caused inflammation was significantly attenuated by A5iOT treatment, such that the CD45+ cell numbers became lower and statistically the same as in the control uninjured group (Figure 4B,C). Moreover, fibrosis was also significantly diminished by A5iOT in the tissues of the old, exercised mice, becoming the same or even slightly lower than in the control mice (Figure 4D,E). Of note, A5iOT worked well for both, males and females in preventing the exercise-related pathologies in the heart and the muscles (Figure S6A–D).

To further examine the effects of administering A5iOT during exercise, we quantitatively assessed the patterns of newly synthesized proteins in muscle and heart, using RayBiotech BONCAT antibody arrays, as described above (Figure 4A). Indeed, UMAPs revealed clear separation of the OE and OEA (old exercised, plus A5iOT) groups, supporting the results on improved tissue health, and a significant downregulation of newly synthesized inflammatory proteins in OEA muscles and heart after the A5iOT treatment (Figures 4F and S6E,F).

Among notable changes, A5iOT downregulated Flt-3 ligand, IL-7R alpha, IL-20R alpha, Epigen, ALK-4, β-catenin, MDC, IL-13R alpha 2, 6Ckine, VEGF-B, TIMP-2, and WIF-1 in old muscles and Fas Ligand, M-CSF, Thrombospondin, TGF-β3, ICK, in old hearts, with the levels of these proteins becoming similar to those of young control mice (Figure 4G). Further, we found that the proteins which are dysregulated by exercise in the old mice and are normalized in these animals by A5iOT, involve interactive Ras/MAPK/Pi3AKT and JAK/STAT pathways (Figure 4H), that are broadly important for tissue health (Romero-Becerra et al. 2020; Moresi et al. 2019; Ghafouri-Fard et al. 2022; Powers et al. 2018).

These results demonstrate the positive effects and potential therapeutic benefits to old tissues and organs of inhibiting TGF-β through A5i and simultaneously providing exogenous oxytocin. When used in combination, A5iOT alleviates inflammation and fibrosis, and youthfully recalibrates de-novo proteomes, thereby negating the negative consequences of exercise in the old. Figure 5 illustrates the overall results of our paper.

3 | Discussion

Exercise exerts a positive influence at the cellular, tissue, and organ levels by reducing genomic instability, cellular senescence, and improving neurological, cardiovascular, and muscle function (Miller et al. 2016). However, exercise is also a stressor

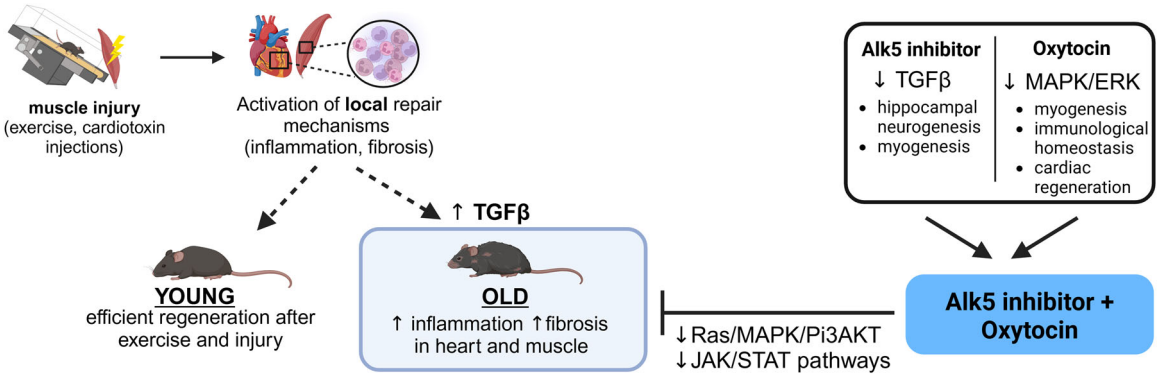

FIGURE 5 | Graphical abstract of the study. Muscle injury and eccentric exercise both lead to repair and regeneration in young animals, but to inflammation and fibrosis in the old, which is associated with excessive TGF-beta signaling. The pathological responses to exercise in old mice were alleviated by administration of Alk5i+ oxytocin, each of which has positive effects on multiple tissues, such as increasing neurogenesis and muscle repair, boosting immune responses, and cardiac regeneration (Elabd et al. 2014; Wasserman et al. 2022; Li et al. 2024b; Pekarek et al. 2020). Combining Alk5i with oxytocin allows lowering the dose of Alk5i, thereby preventing overtly diminished TGF-beta signaling (Mehdipour et al. 2019; Yousef et al. 2015) and enables pathology-free exercise at old age, by youthfully attenuating Ras/MAPK/Pi3Akt and JAKSTAT pathways.

FIGURE 4 | Combination of Alk5 inhibitor and oxytocin alleviates the inflammation and fibrosis brought by exercise in old mice. (A) Schematic of the experimental procedure. Old male and female MetRS mice were injected with A5iOT for 7 days postexercise. (B) Representative immunofluorescent images for macrophages using CD45 in GA and heart. Shown are CD45 (green), laminin (red), and DAPI (blue). (C) Quantification of the number of CD45 positive cells in GA and heart postexercise. (D) Representative trichrome images (magnification × 200) of GA and heart in old mice. (E) Fibrotic index in GA and heart. (F) UMAP plots of the protein expression profiles of old, exercised vehicle-treated (OE) and old, exercised Alk5i +OT-treated (OEA) GA and heart. (G) Heatmap of the 12 GA and 5 heart proteins that are significantly upregulated by exercise and downregulated by Alk5i+OT. (H) STRING network plots showing the protein–protein interaction of DEPs in GA and heart and the enriched pathways. Dashed lines were used to mark the average percentage of CD45-positive cells in young exercise (YE). A different color shade and shape identify individual mouse per group. $N = 4$. Scale bars = 50 μm. Data are mean ± SEM, $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

that increases the levels of pro-inflammatory cytokines in blood plasma (Cerqueira et al. 2020). In our studies, mice underwent eccentric exercise that triggers muscle lengthening under an external force and is expected to improve muscle mass and strength, joint range of motion (Alizadeh et al. 2023), and to provide clinical benefits against cardiovascular diseases (Paluch et al. 2024). Considering EIMD (Li et al. 2024a), we adjusted the duration of treadmill runs to be moderate, as per the observed tolerance of old mice, even though young mice would be able to tolerate higher intensity runs.

Our results established that in the old animals, both skeletal and heart muscle suffered inflammation (more pronounced in males than females) and fibrosis (equally pronounced in both sexes) after the eccentric exercise. These outcomes are counter to the expected health benefits. In fact, the inflammation and fibrosis that were elicited by exercising old mice were similar in their magnitudes to those after muscle injury with cardiotoxin, a potent myonecrotic agent. Perhaps the reluctance to exercise as we age might be a protective mechanism against excessive tissue damage and consequential loss of function. Further clinical studies should be performed to determine the age range when exercise starts to cause more detrimental than beneficial effects, as well as the specific sex-related responses that we noticed in these studies.

In young exercised mice or people, an acute but transient inflammatory response to tissue damage is beneficial for tissue repair by clearing dead or dying cells and contributing to cytokine-promoted myogenesis (Tidball et al. 2021; Kunz and Lanza 2023). However, chronic or excessive inflammation broadly impairs health, maintenance, and repair of muscle (Soliman and Barreda 2022). Chronic inflammation inhibits wound healing systemically and skews immune responses (Li et al. 2023). Muscle and cardiac fibrosis are mainly triggered by the overexpression and accumulation of pro-fibrotic factors (Shen et al. 2005; Gardner et al. 2020; Gallardo et al. 2021). Muscle fibrosis is a major cause of muscle weakness and a hallmark of aging-associated loss of muscle function, dystrophies, and severe injuries (Mahdy 2019). Cardiac fibrosis can lead to cardiac stiffening and impaired cardiac contractility, ultimately leading to heart failure (Jiang et al. 2021).

In search for the mechanism behind the detrimental effects of exercise on old tissues, our BONCAT proteomics, which analyzes 308 cytokines (pro-/anti-inflammatory, pro/anti-fibrotic, pro-/antiangiogenic, and regulators of homeostasis), identified TGF-β pathway, as an age-specific determinant of pathological response of old mice to exercise. TGF-β is a known driver of fibroblast activation and collagen deposition (Meng et al. 2016), as well as a major pro-inflammatory factor (Sanjabi et al. 2009) that is, moreover, a part of the senescence-associated secretory phenotype (Tominaga and Suzuki 2019). Perturbations in TGF-β signaling are implicated in a spectrum of aging-associated diseases, such as diabetes, neurodegenerative disorders, and cancer (Heydarpour et al. 2020; Kashima and Hata 2018; Derynck et al. 2021). At the same time, at its young healthy levels, TGF-β/pSmad signaling is indispensable for productive hematopoiesis and regulation of immune responses, as well as for homeostasis of multiple tissues where this pathway balances cell proliferation and differentiation (Deng et al. 2024).

In addition to TGF-β, overlapping DEPs in heart and GA (e.g., TIMP-2, Ubiquitin, MDC, and Tie-2), all play a major role in inflammation, fibrosis, and immune system activation. TIMP2 inhibits MMPs, leading to ECM accumulation and fibrosis (Arpino et al. 2015). MDC is a chemoattractant for inflammatory cells (NK cells, dendritic cells, lymphocytes) (Korobova et al. 2023). Ubiquitin also participates in inflammatory response and several programmed cell death mechanisms (Cockram et al. 2021). Tie-2 roles in vascular regulation include vascular leakage after injury (Saharinen et al. 2017; Milam and Parikh 2014).

While there are ideas to promote broad rejuvenation with a single factor, and/or to replace exercise with a drug, the multifactorial, multigenetic processes of aging and exercise might require combinatorial approaches. Based on the exercise-specific proteomics, we decided to attenuate TGF-β signaling via Alk-5 inhibitor while simultaneously providing the aging-diminished signaling peptide oxytocin, which was previously reported to have positive effects on cardiac rejuvenation, wound healing, and immunological homeostasis (Elabd et al. 2014; Wasserman et al. 2022; Li et al. 2024b; Pekarek et al. 2020). Adding oxytocin also reduces the required dose of A5i by 10-fold, yet maintains and broadens the positive effects on multiple old tissues, as compared to the use of each drug alone (Mehdipour et al. 2019; Yousef et al. 2015), which, in part, can be attributed to the attenuation of TGF-β pathway by GPCR, through which oxytocin signals (Borroto-Escuela et al. 2022; Chia et al. 2024). We established that A5iOT has a novel pharmacological potential to extend the benefits of exercise at older ages, preventing tissue inflammation and fibrosis and broadly normalizing de-novo proteomes, including the TGF-beta, JAK-STAT, and MAPK pathways. JAK-STAT regulates vascular development and angiogenesis (Xue et al. 2016) and is needed for efficient regeneration (Doles and Olwin 2014); excessive JAK-STAT is associated with upregulation of IL-6 and TNF, inflammatory response (Moresi et al. 2019), rheumatoid arthritis (Thomas et al. 2015), and malignancy (Brooks and Putoczki 2020). The MAPK pathway plays many positive roles that are important for cell viability, proliferation, differentiation, i.e., function and repair of organ systems (Lawrence et al. 2008; Cargnello and Roux 2011). MAPK becomes activated in skeletal muscle after exercise (Kramer and Goodyear 2007); elevated MAPK in the heart is associated with cardiac hypertrophy (Bernardo et al. 2010), which, in turn, is linked to increased interstitial fibrosis, cell death, and cardiac dysfunction. Overall, based on the BONCAT data and the PCA bioinformatics, pro-inflammatory cytokines, which were elevated by exercise in old mice, become attenuated in the presence of A5iOT.

4 | Conclusion

Summarily, in old mice, eccentric exercise exacerbates the key detriments of aging (fibrosis inflammation and perturbation of tissues' proteomes); A5iOT provides health benefits to the animals in exercising at older ages. Before pharmacological prevention of exercise-caused tissue damage is tested for people, fitness assessment, type of exercise, and gradual increase of exercise intensity are prudent to consider, as our study modeled

only eccentric exercises (Cerqueira et al. 2020; Langhammer et al. 2018; Wackerhage and Schoenfeld 2021).

5 | Materials and Methods

5.1 | Study Design

The primary objective of the study was to evaluate the effects of aging in exercise-elicited responses. Our approach was to model EIMD, commonly observed in old people, in vivo using CAG-floxed-Stop-eGFP-mMetRSL274G young (2–4 months) and old (22–24 months) mice, as in (Liu et al. 2017). All procedures were performed in accordance with the Animal Care and Use Committee (ACUC), the administrative panel of the Office of Laboratory Animal Care, UC Berkeley. Young and old mice ($n = 4$ for all treatment groups) underwent exercise or cardiotoxin-injections, injected with azido-nor-leucine treatment (ANL), and treated with either vehicle or Alk5 inhibtor+oxytocin (A5iOT). The levels of inflammation and fibrosis were assessed 7 days post-injury. Exercise-specific changes in de novo proteomes were also analyzed using Click Chemistry and RayBiotech BONCAT Antibody arrays, as (Mehdipour et al. 2019).

5.2 | Exercise-Induced Muscle Injury

Young (2–4 months) (YE) and old (22–24 months) (OE) male and female MetRS mice underwent eccentric exercise programs. They ran on a downhill treadmill at a slope of −14. Before testing, they were trained for 2 days at a low speed of 5–8 m/min for 10 min on Day 1 and 5–10 m/min for 10 min on Day 2. On the third day, mice ran at a start rate of 8 m/min, which was then increased by 1 m/min every 10 min after the 10 min mark. All mice ran until exhaustion or once they reached 60 min. Exhaustion was determined by refusal to run on the treadmill for at least 10 s. All mice were fasted for 2 h before running, with access to water ad libitum. Uninjured young (YC) and old (OC) mice served as controls.

5.3 | Cardiotoxin Injury

Young (2–4 months) (YCTX) and old (22–24 month) (OCTX) male and female MetRS mice were injured by intramuscular injections of cardiotoxin (Sigma C9759, 5 μg per muscle at 0.1 mg/mL) into the tibialis anterior (TA) and gastrocnemius (GA).

5.4 | Azido-Nor-Leucine Treatment

After each injury, mice were injected intraperitoneally with azidonorleucine (ANL) (Jena Biosciences CLK-AA009, 0.02 mmol/kg) daily for 7 days.

5.5 | Alk5 Inhibitor + Oxytocin (A5iOT) Treatment

TGF-β1 Type I Receptor Kinase Alk5 inhibitor 2-(3-(6-Methylpyridin-2-yl)- 407 1H-pyrazol-4-yl)-1,5-naphthyridine (A5i) (Enzo Biosciences 270-445) and oxytocin (OT) (Bachem 4016373.0025) will be combined and administered subcutaneously at 0.02 nmol/g-day and 1 μg/g-day, respectively, for 7 days postexercise, following previously established protocol (Mehdipour et al. 2019). The old and young control groups received Hank's Balanced Salt Solution (HBSS) (Avantor 02-0121-0500) as vehicle control.

5.6 | Tissue and Serum Isolation

Seven days after injury, blood was collected through cardiac puncture using a 1-mL syringe with a 25-gauge needle. Serum was prepared by allowing the blood in 1.5-mL Eppendorf tubes to clot at room temperature for 30 min, followed by centrifugation at 1000 *g* for 5 min at 4°C and collecting the supernatant.

After blood collection, mice were intracardially perfused with 1× PBS. Heart, TA, and GA muscles were then isolated, embedded in OCT (Sakura Finetek, 4583), snap frozen in isopentane cooled to −70°C in a dry ice, and stored at −80°C until analysis.

5.7 | Masson's Trichrome Staining

Masson's Trichrome staining was performed according to the manufacturer's protocol (Abcam, ab150686). Briefly, 8–10 μm heart, TA, and GA sections were fixed with 4% paraformaldehyde (PFA) and preheated Bouin's solution (60°C) for 1 h each. Then, each slide was stained with Weigert's iron hematoxylin for 7 min, Biebrich scarlet-acid fuchsin for 27 min, phosphomolybdic-phosphotungstic solution for 7 min, and aniline blue solution for 30 min, and rinsed in 1% acetic acid solution for 3 min. All sections were dehydrated in 70%, 95%, and 100% ethyl alcohol and cleared in xylene. 2–3 drops of xylene mounting medium were added, and coverslips were placed.

Ten random representative areas with fibrosis were obtained per sample. Fibrotic indices were quantified by dividing total fibrotic area (mm^2) by total tissue area (mm^2) per image. These analyses were performed with ImageJ, and two analysts were blinded to sample identification.

5.8 | Immunofluorescence

Heart, TA, and GA were sectioned (8–10 μm), rehydrated with a wash buffer (1× PBS) for 10 min, fixed with 4% PFA for 10 min, and blocked with 10% serum in PBS for 1 h. They were incubated with primary antibodies (CD45, 1:50, Cell Signaling Technology 70257; Laminin, 1:100, Invitrogen MA106100) at 4°C overnight. The following day, slides were washed with a wash buffer, incubated with secondary fluorochrome-tagged antibodies (AF 488, 1:500, Invitrogen, A21206; AF594, 1:1000 Invitrogen, A11007). The slides were mounted with 2–3 drops of fluoromount-G with DAPI (Invitrogen, 501128966) and viewed under a microscope. IgG controls with isotype-matched antibodies were routinely done and nonspecific fluorescence was

minimal. Ten random representative areas were obtained per sample. % CD45 positive cells were quantified by dividing CD45 positive cells by a total number of nucleus per image × 100. These analyses were performed with ImageJ, and two analysts were blinded to sample identification.

5.9 | Click-Western blot

Serum and protein lysates from heart, TA, and GA were clicked with biotin alkyne (Thermo Fisher Scientific, B10185) using Click-iT® Protein Reaction Buffer Kit (Thermo Fisher Scientific, C10276). To confirm ANL labeling and biotinylation before antibody array experiments, the clicked samples were visualized by performing western blot. Briefly, 10 μL of samples were diluted with 10 μL sample buffer (2X Laemmli buffer with β-mercapthoethanol), heated to 95°C for 5 min, separated through SDS-PAGE on 4%–20% Mini-PROTEAN TGX Precast Gels (Biorad 456-1095), and transferred to 0.45 μm polyvinylidene difluoride membranes (Millipore). The membranes were blocked with protein-free blocking solution (Advansta, R-03023-D20), incubated with peroxidase-labeled streptavidin (KPL, 474–3000) for 1 h, and developed using ECL (Advansta, K-12045-D50). The blots were visualized with a Bio-Rad Gel Doc/ChemiDoc Imaging System.

5.10 | Antibody Array Proteomics

Levels of 308 proteins (L-308 glass array, RayBiotech) were measured in serum, heart, TA, and GA following the manufacturer's protocol. Briefly, clicked serum and protein lysates were dialyzed in 0.2% Triton X-100 in PBS. Alongside, the array membranes were incubated with blocking buffer overnight at 4°C. After blocking, the array membranes were incubated overnight at 4°C with 30 μg of clicked-dialyzed heart, TA, and GA lysates and 400 μg of clicked-dialyzed serum. After washing, the arrays were incubated with Streptavidin-conjugated Cy3 fluorescence dye overnight at 4°C. The array slides were washed, dried, and scanned using GenePix Pro 6.1 software (Molecular Devices). Fluorescence intensities were obtained for each array. Median spot intensities of the raw light intensity data of the arrays were extracted in the R language (R: The R Project for Statistical Computing) with limma, (Ritchie et al. 2015) and were background corrected using the normexp-by-control method (Shi et al. 2010) with reference to the positive and negative controls and an offset of 20. Normalization was done with the recommended method per the L308 user manual, where all samples' expression values were scaled relative to a reference array until all positive controls have the same mean. Raw and normalized BONCAT array data are presented in Data File S1.

Moreover, for each array, detection p-values for proteins were estimated by FDR-controlled (Benjamini et al. 2001) Wilcoxon tests (Wilcoxon 1946) on each protein vs the negative controls, and DEPs identified downstream that lacked p values > 0.05 were disregarded. Hidden batch effects were minimized with supervised surrogate variable analysis (Gagnon-Bartsch and Speed 2012; Buja and Eyuboglu 1992) in the sva package (Leek et al. 2012). Targets on a per-sample basis were then grouped, averaged, and DEPs were identified with the limma-trend approach and a log2FC cutoff of 1. All other array visualizations were produced with the ggplot2 package (Wickham 2009).

5.11 | Network Analysis

GO and KEGG overrepresentation analyses were done using clusterProfiler (Wu et al. 2021) after manually mapping the L308 proteins to their respective genes and entrez-ids in the mouse genome informatics database (Law and Shaw 2018). Enriched pathways were also visualized as circular functional map using the built-in gene-list enrichment function in KOBAS-I (Bu et al. 2021). Only the significant pathways are colored and labeled, with consistent colors used for each pathway KOBAS-i illustration. The STRING database, v12 (http://string-db.org/) (Szklarczyk et al. 2022) was used to search for the relationship between DEPs and construct the PPI network with a combined score over 0.4, which was considered statistically significant. The Markov clustering algorithm, an unsupervised stochastic clustering method for graphs that clusters strongly interconnected nodes, was applied (with an inflation parameter = 3) to the resulting network to identify additional subclusters.

5.12 | Statistical Analysis

Sample sizes were determined by a power analysis to reach at least 85% and the significance threshold (<0.05). The mice were randomized ($n = 4$ per group) and blinded for analysis. All data analyses were either performed using Prism v.8 software from GraphPad, Python, and Microsoft Excel 2019. For comparisons with two or more independent groups and one independent variable, one-way analysis of variance (ANOVA) was used, followed by Dunnett's post hoc test for adjusted P values (significant at an adjusted $p \leq 0.05$). For comparison with two or more independent variables (treatment, age, and sex), two-way ANOVA was used, followed by Sidak's multiple comparisons, which computes for confidence interval and multiplicity adjusted p values (Lee and Lee 2018). When normality was not satisfied, Mann–Whitney tests were performed on comparisons among groups/conditions. Primary data for each figure are reported in Data File S2.

Author Contributions

Joana Marie C. Cruz performed all experiments and generated Figures 1, 2, 3A,C,F and 4A–G,H and Supporting Information Figures 1–3, 5; analyzed and interpreted all the data and produced the figures, co-wrote the manuscript. Hayden Yeung performed the in vivo experiments shown in Figures 1 and 4. Rana Alzalzalee performed experiments on fibrosis and antibody arrays shown in Figures 2A,B and 4D, and Supporting Information Figures 1C, 3. Qile Yang performed the proteomic analysis and generated Figure 3D,E and Supporting Figure 4F,G. Hannaneh Kabir generated Figures 3B, 4F and Supporting Figure 4A–D,I. Samantha Annaliese McDonough performed experiments on inflammation shown in Figures 1B,D and 4B, Supporting Information Figures 1A and 2B,C,E. Xiaoyue Mei provided Supporting Information Figure 4H. Michael J. Conboy planned and directed the study and co-wrote the manuscript. Irina M. Conboy

planned, directed, integrated, and interpreted the study and co-wrote the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data associated with this study are present in the paper or the Supporting Materials.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.