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Chymase-dependent production of angiotensin II: an old enzyme in old hearts

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Running head: RAS signaling in aged and age-exercised rats

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Abstract (249**)**

Age-dependent alteration of the renin-angiotensin-system (RAS) and generation of angiotensin II (Ang II) are well documented. By contrast, RAS-independent generation of angiotensin II in aging and its responses to exercise have not been explored. To this end, we examined the effects of chymase, a secretory serine protease, on the angiotensin converting enzyme (ACE)-independent conversion of Ang I to Ang II. We hypothesize that age-dependent alteration of cardiac Ang II formation is chymase-dependent in nature and is prevented by exercise training. Experiments were conducted on hearts isolated from young (3-month), aged (24-month), and aged rats chronically exercised on a treadmill. In the presence of low Ang I levels and downregulation of ACE expression/activity, cardiac Ang II levels were significantly higher in aged than young rats, suggesting an ACE-independent response. Aged hearts also displayed significantly increased chymase expression and activity, as well as upregulation of tryptase, a biological marker of mast cells, confirming a mast cell-sourced increase in chymase. Coincidently, cardiac superoxide produced from NADPH oxidase (Nox) was significantly enhanced in aged rats and was normalized by exercise. Conversely, a significant reduction in cardiac expression of ACE2 followed by lower Ang 1-7 levels, and downregulation of the Mas receptor (binding protein of Ang 1-7) in aged rats was completely reversed by exercise. In conclusion, local formation of Ang II is increased in aged hearts and chymase is primarily responsible for this increase. Chronic exercise is able to normalize the age-dependent alterations via compromising chymase/Ang II/AT1R/Nox actions, while promoting ACE2/Ang 1-7/MasR signaling.

New & Noteworthy

Aging increases ACE-*independent* production of cardiac Ang II, a response that is driven by chymase in an exercise-reversible manner. These findings highlight chymase, in addition to ACE, as an important therapeutic target in the treatment and prevention of Ang II-induced deterioration of cardiac function in the elderly.

Key Words: aging; chymase; angiotensin converting enzyme; angiotensin II; exercise

Introduction

Angiotensin II (Ang II) is the major biologically active molecule of the renin-angiotensin system (RAS), which is involved in the homeostasis of blood pressure through its effects on water and electrolyte balance, as well as peripheral vascular resistance (46). Ang II is produced from the decapeptide angiotensin I (Ang I) via cleavage of two c-terminal amino acids by angiotensin converting enzyme (ACE). When bound to the angiotensin type 1 receptor (AT1R) on the vasculature, Ang II initiates vasoconstriction, whereas it elicits NO-mediated vasodilation via binding to the endothelium-located AT2R (18). Alternatively, cleavage of Ang II by angiotensin converting enzyme 2 (ACE2) produces the heptapeptide angiotensin 1-7 (Ang 1-7), which when bound to the Mas receptor (MasR) (48), initiates downstream vasodilator activity that can counteract the hypertensive effects of AT1R signaling. Ang 1-7 therefore possesses cardioprotective properties (9), through effects that can prevent and/or reverse heart failure and blunt hypertensive cardiac remodeling (3; 38; 43). In addition to the systemic RAS, local RAS has been found in many tissues, including the heart, which functions both independently and in correlation with systemic RAS components (1; 9; 44). Importantly in the heart, there is an alternate pathway for Ang II synthesis by the endopeptidase chymase, a serine protease that is predominantly found in granulocytes called mast cells located within the interstitium. Mast cells contain granules that are packaged with cytokines and proteases (including chymase) that are released during the inflammatory process via a classically mediated lingand-dependent pathway (9; 13; 55). Chymase acts in much the same manner as ACE but with a 20-fold higher catalytic activity for the conversion of Ang I to Ang II (2). In this context, chymase is believed to serve as a major Ang II-forming enzyme in the human heart (21; 22), and is reported to be involved in many pathological processes such as hypertension, atherosclerosis, vascular proliferation,

development of cardiomyopathies, myocardial infarction, heart failure as well as cardiac fibrosis (20; 21; 25; 31; 54; 61).

Ultimately, the impact of altered RAS signaling in the cardiovascular system extends beyond just the control of vascular tone, and actually significantly contributes to the pathogenesis of a variety of cardiovascular diseases including aging-induced cardiac dysfunction (14). During the process of aging, chronic elevation of Ang II evokes generation of reactive oxygen species (ROS) via binding with AT1R to activate NADPH oxidase (Nox) (11; 60). This Ang II-AT1R–Nox signaling cascade may in turn, trigger downstream stress- or inflammatory-related signaling to further produce ROS (57). Alternatively, oxidative stress *per se*, can serve as a trigger to stimulate the release of chymase from mast cells, via a non-ligand-mediated signaling pathway (32), leading to the formation of a positive feedback loop during the process of Ang II stimulation of oxidative stress, and *vice verse*. Interestingly, regular exercise has been reported to reduce age related increases in tissue oxidative stress and inflammation (4) and demonstrated to be cardioprotective in both pathological and physiological conditions (26; 52; 53). One of the underlying mechanisms involved in the protective actions of exercise is via the potentiation of the ACE2/Ang1-7/MasR pathway that can balance or even outweigh actions of the ACE/Ang II/AT1R/ROS signaling in modifying acute and chronic inflammation, fibrogenesis, and cellular proliferation (14; 26).

The pathological significance of changes in systemic RAS is well established (24), however the mechanisms responsible for aging-dependent changes in cardiac Ang II signaling, that are specifically driven by chymase, are much less understood. One study found an approximately 20% increase in total mast cell number with greater than a 4-fold increase in activated mast cells in aged lymphatic tissue (10), findings that are important since chymase is

packaged in, and released from mast cells. To date, neither the roles of chymase in age-dependent cardiac formation of Ang II, nor changes in chymase activity in response to exercise have been reported in the literature. Given chymase as an important Ang II synthase in the heart, along with the lack of studies focused on the pathological significance of chymase-sourced Ang-II during cardiac aging, we hypothesized that chymase is a key contributor to the age-dependent increase in cardiac formation of Ang II, a response that we posited can be prevented by exercise training via compromising chymase actions and recruiting ACE2/Ang1-7/masR signaling. We specifically focused on the association between cardiac aging and chymase action by determining chymase-dependent formation of Ang II in myocardium of young and aged rats.

Materials and Methods

Animals: 3 month (Young)- and 20 month-old male Fischer 344 rats were obtained from Charles River Laboratories. Aged rats were randomly divided into 2 groups of sedentary and exercised rats, and housed for an additional four months to yield 24 month-old sedentary (Aged) and 24 month-old exercised (Aged-Ex) groups. Protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current guidelines of the National Institutes of Health and American Physiological Society for the care and use of laboratory animals.

Exercise Training: Rats were exercised on a treadmill (model 4215; Quinton Instr. Co) starting at 20 months of age. As described previously (52), a jet of air and a mild electrical tail shock stimulus were used to keep the rats running on the treadmill, but caused no injuries to the rat tail. The exercise protocol was similar to that used in our previous studies (52; 53). Exercise activity on the treadmill was carried out for 5 days/week for 4 months. The length of time on the treadmill was initially 5 min/day and was progressively increased to a maximum of 60 min/day by the end of the sixth week. The speed and angle of the treadmill was increased from 11 m/min at a 0° grade on day 1, to a maximum of 38 m/min at a 5° grade by the end of the sixth week. All parameters were subsequently kept at a maximal level until the end of the exercise period. Sedentary rats were handled daily on the treadmill without running. At the end of exercise training, rats were anesthetized with isoflurane and euthanized. Following bilateral thoracotomy, the heart was excised and immediately pulverized in liquid N_2.

130 *Measurement of angiotensin peptides*: Ang I, Ang II, and Ang 1-7 peptides in cardiac tissue
131 were measured by ELISA (Mybiosource INC, San Diego, CA). Based on the instructions
132 provided by the manufacturer, pulverized whole hearts were homogenized in a sample buffer,
133 centrifuged at 1,000 xg for 10 minutes and the supernatant fractions were collected. In a 96-well
134 plate, 50 µl of samples or peptide standard solution was added to each well, followed by the
135 addition of 100 µl of horseradish peroxidase (HRP)-conjugate reagent. The plate was gently
136 mixed, covered with a plastic membrane, and incubated at 37^0C for 60 minutes. Following
137 incubation, the plate was washed with wash buffer four times. The plate was subsequently
138 blotted dry, and 50 µl of each Chromogen Solution A and Chromogen Solution B were added to
139 each well. The plate was gently mixed and incubated for an additional 15-min at 37^0C, and 50 µl
140 of stop solution was then immediately added. The absorbance was read at 450 nm using a
141 SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The
142 amount of angiotensin peptides was calculated by comparing absorbance readings of samples to
143 those of the standard curve. Values were normalized to mg of tissue protein.
144
145 *Enzymatic Activity*: Nox activity was measured using an established lucigenin detection analysis
146 (28). Liquid N_2 pulverized hearts were homogenized in phosphate buffer containing 20mM
147 monophasic potassium phosphate KPO_4, 1 mM EDTA and protease inhibitor cocktail. Samples
148 were centrifuged at 1000 g, the supernatant was aspirated and protein content was determined
149 using the Bradford method (Bio-Rad, Hercules, CA). The reaction was conducted in a final
150 volume of 1 mL, which contained reaction assay buffer (50 mM KPO_4, 150 mM sucrose, and 1
151 mM EDTA pH 7.0), 5 µM lucigenin, and 100 µM NADPH. The reaction was initiated by the
152 addition of 50µl of homogenate (approximately 200 µg protein) that was pre-incubated with or

without 100 μM apocynin (inhibitor for Nox). The chemiluminescence generated by the reaction was recorded for two minutes by scintillation counter (LS6000IC, Beckman Instruments). Nox activity was calculated by subtracting the value of apocynin-treated fraction from total values (without apocynin), and reported as CPM (counts/min)/μg protein after background subtraction.

Activities of ACE and ACE2 were determined using specific fluorometric substrates Abz-FRK(Dnp)-P and Mca-APK(Dnp) (Enzo Life sciences, Farmingdale, NY) respectively (19). Hearts were homogenized in 0.1 M Tris-HCl buffer containing 50 mM NaCl (pH 7.0) for ACE and 0.2 M Tris-HCl containing 200 mM NaCl (pH 7.5) for ACE2. Samples were centrifuged at 1000g for 10 min and the supernatant fraction was collected for analysis. Activity assays were conducted in the same buffers with additional 10 μM $ZnCl_2$. 10 μM of the appropriate fluorogenic substrate was added to approximately 40 μg protein samples in a final reaction volume of 100 μl. The hydrolytic rate of either fluorogenic substrate was determined continuously for 40 min by incrementally reading fluorescence at 320 nm excitation and 420 nm emission. In separate experiments, the enzymatic specificity was validated by testing the reaction in the presence of 10^{-6} mol/l captopril (ACE inhibitor) or DX600 (ACE2 inhibitor). Protein concentration of samples was measured by the Bradford method (Bio-Rad, Hercules, CA) and used to normalize detected tissue fluorescence. The maximum rate of reaction (Vmax) was defined as changes in relative fluorescence unit (RFU) per unit time (minute). The enzyme activity was expressed as Vmax per μg protein.

Chymase activity was determined by the rate of cleavage of the chromogenic substrate Suc-AAPF-pNA (Enzo Life Sciences) (39). Specifically, hearts was homogenized in the assay buffer containing 125 mM $NaCl_2$, 10 μM $ZnCl_2$ and 25 mM HEPES (pH 7.4) with addition of 0.01% triton X-100 (22). After homogenization, samples were centrifuged at 1,000 xg for 10

176 min, the supernatant fraction was collected and protein content was determined using the
177 Bradford method. Approximately 50 µg of protein were pre-incubated with or without 100 µM
178 chymastatin (chymase inhibitor) in assay buffer for 5 min, followed by incubation with 4 mM
179 Suc-AAPF-pNA at 37^0C in a total reaction volume of 250 µl. Spectrophotometric determination
180 of absorbance at 405 nm was started immediately after adding Suc-AAPF-pNA and continued
181 for 60 min. Chymase activity was calculated by subtracting the value of chymastatin-treated
182 fraction from total values (without chymastatin), and presented as Vmax per mg protein.
183
184 *Western blot analysis*: Isolated hearts were pulverized in liquid N_2. Equal amounts of total
185 protein (50 µg) extracted were loaded onto and separated by 10% SDS-PAGE gel and transferred
186 to a PVDF membrane. The membrane was probed with specific primary antibodies for ACE
187 (1:200 dilution), ACE2 (1:500), chymase (1:200), mast cell tryptase (1:1000), and MasR (1:500)
188 purchased from Abcam (Cambridge, MA), AT1R (1:1000 sc-1173) and AT2R (1:500) obtained
189 from Santa Cruz (Santa Cruz Biotechnology Inc, CA), and GAPDH (1: 2500) from Millipore
190 (Billerica, MA), followed by appropriate secondary antibodies conjugated with horseradish
191 peroxidase (1:2000-1:10,000). Specific bands were visualized with a chemiluminescence kit
192 (Thermo Scientific, Rockford, IL) and normalized to GAPDH. The X-ray film was scanned into
193 a computer and band densitometry was digitalized with UN-SCAN-IT software (Silk Scientific,
194 Orem, UT).
195
196 *Calculation and statistical analysis*: Data are expressed as means ± SE, and n refers to the
197 number of rats. Tests of data normality were conducted following which, one-way ANOVA or

198 the Kruskal Wallis test was performed where appropriate. Statistical significance was accepted at
199 a level of $p<0.05$.
200

Results:

Aging induced imbalance between angiotensin II and angiotensin 1-7 axes

Cardiac angiotensin peptide contents are depicted in Figure 1. Cardiac tissue levels of Ang I were significantly lower in aged than in young control animals (Figure 1a), findings that are consistent with most previous studies (16). Alternatively, Ang II in hearts of aged animals was significantly increased compared to that in young controls (Figure 1b) while the levels of the cardioprotective peptide, Ang 1-7, were markedly reduced (Figure 1c). The age-dependent changes in angiotensin peptides were normalized by exercise activity (Aged-Ex).

Aging-dependent alterations/reductions of ACE and ACE2 in an exercise-reversible manner

Expression of ACE and its activity are summarized in Figure 2. Interestingly, ACE expression (Figure 2a) and activity (Figure 2b) were reduced significantly in aged compared to young rats, implying that the age-induced increase in Ang II (shown in Figure 1b) appears to be independent of ACE. Moreover, exercise training prevents the age-related reductions in both ACE expression and activity. In separate experiments, ACE activity was tested in the presence of captopril (10^{-5} mol/l; specific inhibitor of ACE) in order to validate the specificity of the enzyme. As shown in Figure 2c, cardiac ACE activity in the three groups of rats was eliminated by captopril, confirming the specificity of the response.

ACE2 expression and activity are summarized in Figure 3. As shown in panel (a), the age-associated downregulation of cardiac ACE2 was not only normalized, but upregulated by exercise training. In similar fashion, the pattern of decreased ACE2 activity was completely reversed by exercise training to levels observed in control animals (Figure 3b). The specificity of

ACE2 was also validated by experiments, in which DX600 (10^{-5} mol/l; specific inhibitor of ACE2) abolished the enzyme activity in all groups of rats (Figure 3c).

Age-induced increase in chymase expression/activity paralleled with changes in mast cell content

In order to clarify the nature of the ACE-independent increase in cardiac Ang II in aged rats (Figures 1 and 2), cardiac chymase expression and activity were evaluated and are presented in Figure 4. Contrary to the downregulation of ACE expression and attenuation of ACE activity (Figure 2a-2b), aged rats display an upregulation of chymase expression (Figure 4a), accompanied by a significant increase in chymase activity (Figure 4b). To identify the source of chymase in the cardiac tissue, mast cell tryptase expression was used as an indirect determinant of mast cell content, based on the fact that the tryptase is highly specific for mast cells and its release is proportional to the activation of mast cells (45). As shown in Figure 4c, changes in tryptase expression, characterized as an age-dependent upregulation was paralleled with those of chymase expression/activity (Figure 4a-4b), confirming the specific correlation between the two enzymes. Both increments in chymase and tryptase were presented in an exercise-reversible manner, even though their protein expression did not fully recover to levels observed in young rats (Figure 4a). Thus, the age-dependent increase in cardiac Ang II appears to be a chymase-driven response (Figure 4).

Reciprocal potentiation between oxidative stress and chymase-Ang II

To evaluate roles of superoxide in the age-induced altered Ang II signaling, Nox activity was assessed. In correlation with changes in chymase, tryptase and Ang II, Nox activity was

246 significantly elevated in aged compared to young hearts, a response that was also present in an
247 exercise-reversible manner (Figure 5), revealing the specific linkage amongst them.
248
249 ***Different responses of angiotensin receptors to aging and exercise***
250 Protein expressions of downstream targets of Ang II and Ang 1-7, namely AT1R, AT2R
251 and MasR are presented in Figure 6. In the presence of comparable expression of cardiac AT2R
252 among all groups (Figure 6b), cardiac expression of AT1R was upregulated in aged compared to
253 that in young rats (Figure 6a). The increased expression of AT1R was not significantly changed
254 by exercise training, which implies an age-intensifying action of Ang II. However, the age-
255 induced downregulation of MasR was normalized by exercise training (Figure 6c), suggesting an
256 exercise-favorable action on the beneficial Ang 1-7 cascade.
257 Taken together, our data unravel an ACE-*independent* chymase-mediated increase in
258 cardiac Ang II, as a function of age, a response that was prevented by exercise activity via
259 suppression of chymase-dependent Ang II production accompanied with decreases in oxidative
260 stress, and recruitment of ACE2-mediated Ang II degradation.
261

Discussion

The salient findings of the present study are that 1) the age-dependent increase in cardiac Ang II is independent of ACE activity and is a chymase-driven response. Thus, elevation of Ang II levels in aged hearts is proportional to chymase expression/activity and superoxide production, and correlates inversely with ACE activity. 2) Suppression of ACE2 expression and activity contributes significantly to reduced formation of Ang 1-7 in aged hearts which is coupled with 3) a concurrent downregulation of MasR and upregulation of AT1R, thus creating an imbalance between the chymase/Ang II/AT1R/Nox and ACE2/Ang1-7/MasR axes of the RAS. A decrease in the ACE2/Ang-(1-7) cascade diminishes its ability to counterbalance the vasoconstrictor/hypertrophic/proliferative effects of Ang II. 4) Chronic exercise of aged rats results in a decrease in chymase activity and suppression of oxidative stress, enables recovery of ACE and ACE2 activity to enhance Ang 1-7 levels, and normalizes MasR expression. As such, the overall effect of exercise re-establishes balance between the two opposite pathways (Figure 7). Taken together, our studies clarified for the first time, the pathological significance of chymase in the age-dependent alteration of cardiac Ang II signaling, an impairment that is reversible by chronic exercise training. Our results may serve as an explanation, at least in part, for the inadequate outcomes that result from long-term use of renin and ACE inhibitors compared to other antihypertensive classes (21; 37; 47). Moreover, our findings shed light on the clinical interventions that give merit to chymase as a therapeutic target, instead of the current use of ACE inhibitors alone, to reverse the adverse Ang II profile in aged populations.

Potentiation of Chymase/Ang II/AT1R/Nox signaling, as a function of age

The local RAS in cardiomyocytes and coronary vessels is propelled by the *de novo* synthesis of Ang I from renin, followed by conversion of Ang I to Ang II through the rate-limiting cleavage step by ACE (40; 44). Alternatively, an endopeptidase, chymase, has been identified in human and animal hearts and constitutes a non ACE-related pathway for Ang II production (21; 56). In this regard, chymase is believed to play a pivotal role in Ang II formation in human hearts (55). The contribution of chymase to pathophysiological changes in the cardiovascular system has attracted considerable attention, based on observations that long-term treatment of patients with ACE inhibitors is associated with a phenomenon known as "angiotensin II escape", which is characterized as the rebound of circulating Ang II to pretreatment levels (36). Additionally, evidence exists that show not all cardiac Ang II formation and action are sensitive to angiotensin receptor blockers (ARBs) or ACE inhibitors (6; 15; 23). Moreover, during the process of angiotensin signaling, local modifications/alterations in the synthesis of Ang II can result from changes in activity and structure of RAS enzymes; such changes can exacerbate with age-related excess of local Ang II (30). Reports show that cardiac-derived Ang II mediates cardiomyocyte remodeling. This remodeling is responsible for age-dependent cardiac dysfunction and heart failure observed in transgenic mice that develop Ang II-mediated cardiac hypertrophy in the absence of hypertension (17). In this context, the nature of tissue-specific formation of Ang II as a function of aging formed the basis of the present study, which was aimed to extend our current understanding of age-dependent changes in cardiac RAS components and alternative formation of Ang II. Indeed, our results indicate that aged hearts exhibit a significantly high level of Ang II (Figure 1b), a finding that is paradoxically coupled to declines in its precursor (Ang I; Figure 1a) and its rate-limiting enzyme (ACE; Figure 2). This negates the possibility that the response is ACE-dependent and challenges a generally accepted

concept that the increased level of Ang II is due exclusively, to elevated ACE activity. As such, upregulation of chymase expression and greater chymase activity in aged hearts (Figure 4) reveal a strong causal relationship between the enzyme action and formation of its product (Ang II), thus supporting chymase as the primary source for cardiac Ang II in aged rats.

Since mast cells are the predominant producers of chymase in heart tissue, the result of increases in cardiac mast cells, as indicated by upregulation of tryptase (Figure 4c) provides molecular evidence for the mast cell-sourced increase in chymase, as a function of age. Furthermore, the augmentation of superoxide in aged hearts (Figure 5) is able to trigger a shift of mast cells from a non-activated to activated state, resulting in the release of more chymase (32; 33) to further generate Ang II. Specifically, the activated mast cell is defined as the cells that have previously been matured by either binding with their immunoglobin ligands, or through non-ligand mediated pathways, such as by ROS, and are ready for degranulation (release of their granules) in response to the next stimulus (13; 32). Consistent with our findings, an association between aging and increases in mast cell number and activation has also been reported (10). In regards to whether ROS serves as an initiator to stimulate release of chymase, or as a consequence of activation of chymase/Ang II/Nox signaling, this issue remains undetermined, however, we believe that oxidative stress and activation of chymase act as reciprocal causations during the pathogenesis of age-specific alteration of Ang II signaling (Figure 7a).

One study has also demonstrated a switch from ACE-*dependent* to ACE-*independent* formation of Ang II during progression of metabolic syndrome, an age-dependent metabolic disorder. Accordingly, diabetic kidneys exhibit vasoconstriction in response to exogenous administration of Ang I. This response was sensitive to inhibitors of serine protease (chymase) but resistant to those of ACE. An effect directly opposite to this was observed in kidneys of

control mice (41). Thus, the evidence for chymase-dependent accumulation of Ang II in aged hearts not only offers an explanation for the increase in Ang II paradoxically associated with reductions in its precursor (Ang I) and ACE, but it additionally indicates a pathological involvement of chymase in age-specific Ang II-generation via a distinctly different pathway from that of the canonical ACE-dependent pathway.

Age-dependent compromise of the ACE2/Ang-1-7/MasR axis

ACE2 acts on Ang II to produce Ang 1-7 that binds to MasR and evokes protective properties to counterbalance ACE/AT1R-dependent detrimental activity (48). Since the discovery of ACE2 over a decade ago (18), a balance between these two opposing pathways (ACE vs. ACE2) has served as an important parameter in pathophysiological evaluation of RAS actions (42). In this context, not only the Ang II/ACE/AT1R axis, but also the ACE2/Ang 1-7/MasR arm merits consideration as regulatory targets in governing the function of RAS (58). In accordance with this concept, altered actions of both pathways have been linked to a variety of cardiovascular pathologies, including age-related cardiac dysfunction. For instance, renal ACE2 gene expression was downregulated in multiple hypertensive models (12). Moreover, heterozygous ACE2-deficient animals were susceptible to heart diseases (59), and homozygous ACE2-knockout mice developed an age-dependent cardiomyopathy with an accumulation of cardiac Ang II (12). On the other hand, myocardial infarction-induced heart failure and ischemic cardiomyopathy were reported to be alleviated significantly in response to administration of Ang 1-7 (5; 35). Consistent with these reports, we also indicate an age-dependent compromise of the protective signaling, characterized as significant suppression of ACE2 expression and activity (Figure 3), followed by a decrease in cardiac Ang 1-7 (Figure 1c) and concomitant association

with a more than two-fold downregulation of MasR (Figure 6c) in aged compared to young rats. Results similar to these are observed in type II diabetic mice (41). ACE2, which exhibits the highest catalytic efficiency of any of the RAS enzymes, has emerged as a crucial regulator of cardiac function and blood pressure, based on its ability to hydrolyze Ang II to Ang 1-7 (43). This notion of ACE2 serving as an Ang II clearance enzyme is supported by evidence that inactivation of ACE2 is correlated with a deficiency in plasma Ang II clearance following a bolus injection of Ang II (29). As such, a decline in ACE2 and ACE participates reciprocally with the increase in chymase action, in the accumulation of Ang II with concomitant attenuation of Ang 1-7 in aged hearts. With regard to the potential use of ACE inhibitors and ARBs in improvement of physical function among older adults (8), several studies have improved our basic understanding of critical pathophysiological factors that modulate the RAS to improve cardiac function. It is important to note, however, that the long-term therapeutic efficiency of ACE inhibitors to restore age-related decline in cardiovascular function is somehow, weak at best (21; 37; 47). Nevertheless, favorable outcomes are observed in the elderly patients treated with ARBs (34; 49). This phenomenon can be mechanistically explained, at least in part, by our findings that show 1) the involvement of the ACE-independent Ang II pathway and 2) an upregulation of AT1 and downregulation of Mas receptors, as a function of aging.

Exercise balances the age-induced shifts between Chymase/Ang II/AT1R and ACE2/Ang-1-7/MasR

Accumulating evidence show that exercise favors a shift in the RAS toward the ACE2/Ang 1-7/MasR pathway in both normal and diseased hearts (7; 19; 62), skeletal muscles (27) and kidneys (50). To the best of our knowledge however, this is the first study that indicates

an age-specific chymase-dependent increase in Ang II that is reversed by exercise training. Our findings show a phenotype of exercise-induced optimal regulation of the ACE2/Ang 1-7/MasR axis that is identical to those reported previously. This phenotype is characterized by the potentiation of ACE2 activity (Figure 3), restoration of Ang 1-7 levels (Figure 1c) and increase in expression of MasR (Figure 6c), leading to a reduction in Ang II (Figure 1b) and suppression of superoxide (Figure 5). Nonetheless, the novel finding presented in the present study is that increased exercise, independent of aging, can enhance ACE activity (Figure 2) and decrease chymase activity (Figure 4). As such, the exercise-induced normalization of ACE (increased) and chymase (decreased) work in concert with the upregulation of ACE2 to shift the RAS pathway away from Ang II production toward production of Ang 1-7. This shift is accomplished by favoring ACE2-mediated degradation of Ang II to Ang 1-7, and Ang I to Ang 1-9, the latter of which is subsequently converted by the corrected ACE activity to further increase Ang 1-7 content in the tissue. In addition, even in the absence of significant effects on AT1R (Figure 6a), exercise can still amplify Ang 1-7-mediated cardioprotective responses (Figure 7) through the upregulation of downstream MasR expression (Figure 6c).

Perspectives and Alternatives

The literature is replete with studies that highlight a residual risk of cardiovascular events in patients who are treated with RAS inhibitors, a phenomenon that has been attributed to insufficient inhibition of Ang II synthesis or incomplete blockade of intracellular-based actions of Ang II (51). Pharmacological approaches that prevent Ang II synthesis, inhibit ACE activity, and block the peptide binding to AT1R epitomize the foundation of current treatment and prevention strategies against heart diseases. However, these therapies fail to effectively inhibit

the intracellular formation or activity of Ang II. Thus, the broader implication of the present study, which shows an age-specific increase in chymase-dependent Ang II production, is that ACE inhibitors alone may not be sufficient for preventing cardiac functional decline in aged populations; they may require combinatorial treatment with both ACE and chymase inhibitors to optimally protect the elderly heart from Ang II-induced deteriorations. On the other hand, while this study indicates a correlation between cardiac chymase and age, it has not yet provided a functional link between the chymase-dependent formation of Ang II and changes in cardiac function, or explored possible mechanisms responsible for the exercise-dependent modulation of functional changes via providing chymase inhibitors; these issues will be clarified in our future studies.

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Figure Captions:

Figure 1: Cardiac levels of Ang I (a), Ang II (b) and Ang 1-7 (c) in young and aged rats, and aged rats with exercise training (Aged-Ex) (n=5-6 in each group). *Significant difference from young group. #Significant difference from aged group.

Figure 2: (a) Protein expression of ACE (3 blots) and (b) ACE activity (n=6 in each group) in cardiac tissue of young and aged rats, and aged rats with exercise training (Aged-Ex). (c) ACE activity in the three groups, represented as changes in relative fluorescence unit (RFU) over time (minute) in the presence (+) and absence (-) of captopril (10^{-5}M), a specific inhibitor of ACE (n=6 in each group). *Significant difference from young group. #Significant difference from aged group. +Significant difference between two curves.

Figure 3: (a) Protein expression of ACE2 (3 blots) and (b) ACE2 activity (n=5) in cardiac tissue of young and aged rats, and aged rats with exercise training (Aged-Ex). (c) ACE2 activity measured in the presence (+) and absence (-) of DX600 (10^{-5}M), a specific inhibitor of ACE2 (n=6 in each group). *Significant difference from young group. #Significant difference from aged group. +Significant difference between two curves.

Figure 4: (a) Chymase protein expression (3 blots) and (b) activity (n=5-6), and (c) tryptase protein expression (2 blots) in cardiac tissue of young and aged rats, and aged rats with exercise training (Aged-Ex). *Significant difference from young group. #Significant difference from aged group.

Figure 5: NADPH oxidase (Nox) activity (indicative of superoxide production) in cardiac tissue of young, aged, and aged rats with exercise training (Aged-Ex) (n=5-6 for each group). *Significant difference from young group. #Significant difference from aged group.

Figure 6: Protein expression of AT1R (a), AT2R (b) and MasR (c) in cardiac tissue of young and aged rats, and aged rats with exercise training (Aged-Ex) (3 blots for each receptor). *Significant difference from young group. #Significant difference from aged group.

Figure 7: Schematic illustration of changes in cardiac angiotensin signaling, as a function of aging and exercise. (a) Aging hearts exhibit reductions in Ang I, ACE, ACE2, and MasR, whereas upregulation of chymase and AT1R, leading to significant increase in Ang II, paralleled with increases in superoxide that in turn, stimulates chymase-derived Ang II, and attenuation in Ang 1-7. (b) Exercise training primarily reverses all of the alterations, via suppressing chymase and superoxide, whereas normalizing actions of ACE, ACE2, and MasR. (c) As a result, the age-dependent imbalance between the Ang II and Ang 1-7 axes is primarily corrected by exercise training. (+) indicates a stimulation; (-) indicates an inhibition.

ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2; Ang II: angiotensin II; AT1R: angiotensin type 1 receptor; AT2R: angiotensin type 2 receptor; MasR: Mas receptor; Nox: NADPH oxidase; ROS: Reactive Oxygen Species.

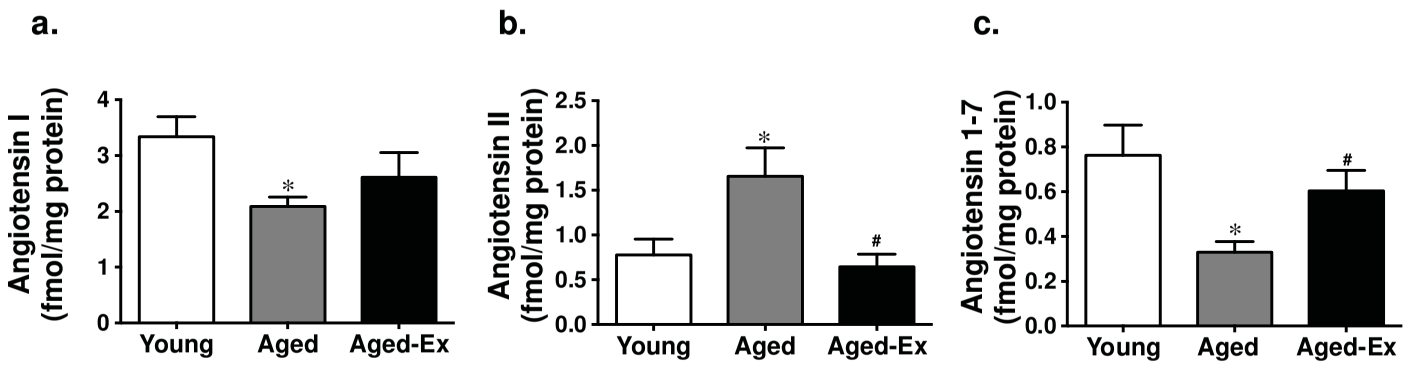
a.
Angiotensin I
(fmol/mg protein)
4
3
2
1
0
*
Young
Aged
Aged-Ex
b.
Angiotensin II
(fmol/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Young
Aged
Aged-Ex
c.
Angiotensin 1-7
(fmol/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Young
Aged
Aged-Ex

Figure 1

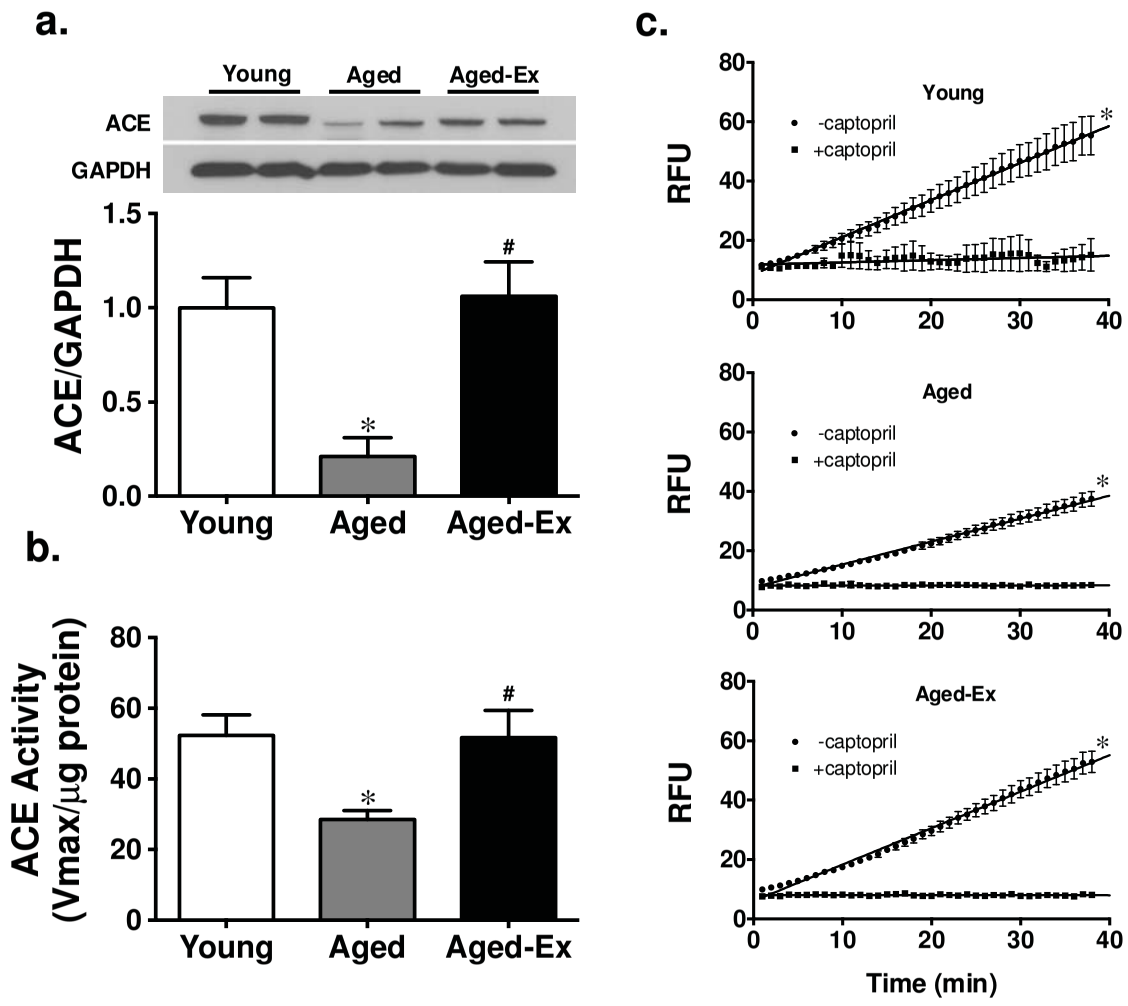
a.
Young
Aged
Aged-Ex
ACE
GAPDH
ACE/GAPDH
1.5
1.0
0.5
0.0
*
#
Young
Aged
Aged-Ex
b.
ACE Activity
(Vmax/µg protein)
80
60
40
20
0
*
#
Young
Aged
Aged-Ex
c.
Young
-captopril
+captopril
RFU
80
60
40
20
0
0
10
20
30
40
*
Aged
-captopril
+captopril
RFU
*
Aged-Ex
-captopril
+captopril
RFU
*
Time (min)

Figure 2

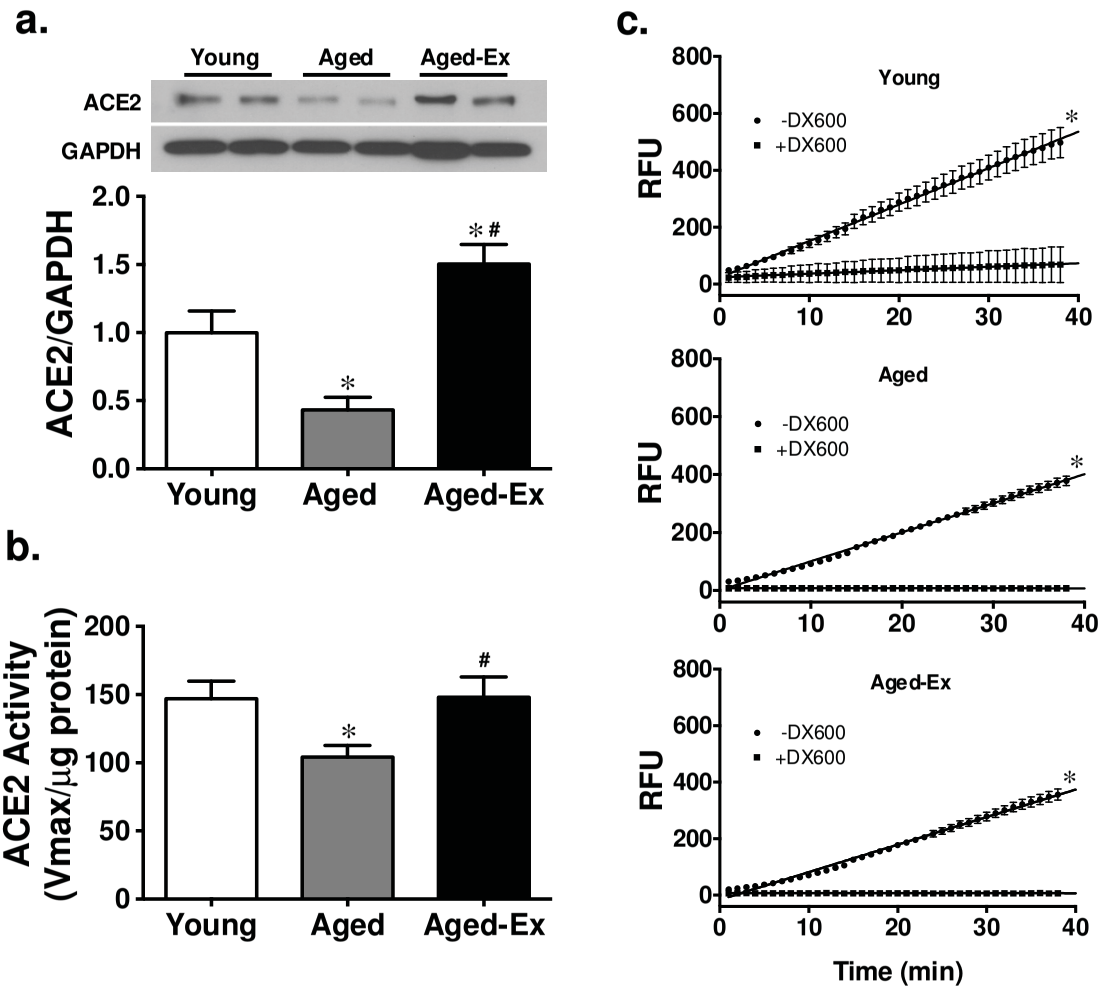
a.
Young
Aged
Aged-Ex
ACE2
GAPDH
ACE2/GAPDH
2.0
1.5
1.0
0.5
0.0
*#
*
Young
Aged
Aged-Ex
b.
ACE2 Activity
(Vmax/μg protein)
200
150
100
50
0
#
*
Young
Aged
Aged-Ex
c.
Young
-DX600
+DX600
RFU
800
600
400
200
0
*
0
10
20
30
40
Aged
-DX600
+DX600
RFU
*
Aged-Ex
-DX600
+DX600
RFU
*
Time (min)

Figure 3

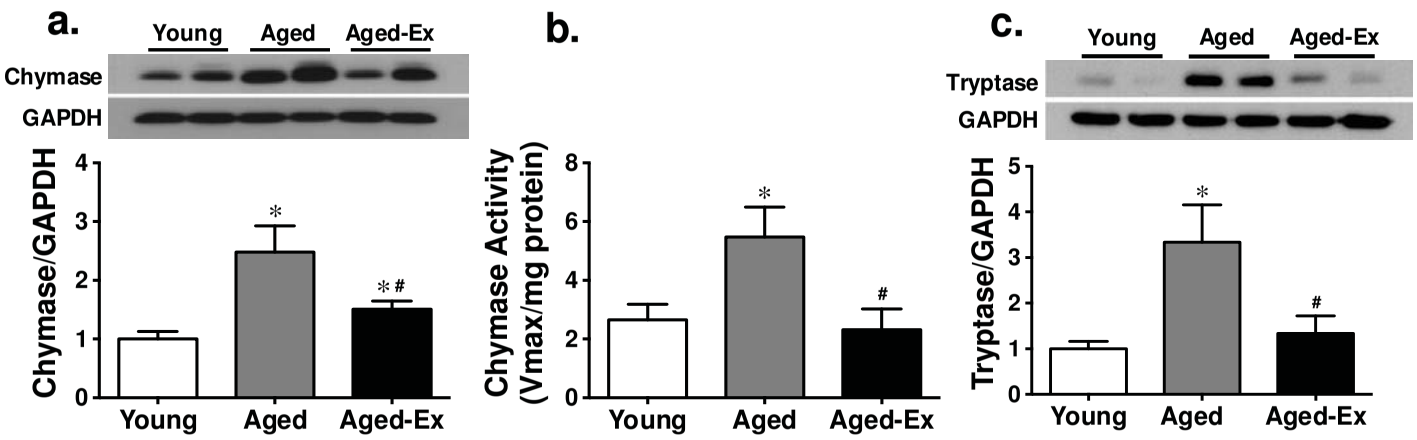
a.
Young
Aged
Aged-Ex
Chymase
GAPDH
Chymase/GAPDH
0
1
2
3
4
*
*#
Young
Aged
Aged-Ex
b.
Chymase Activity
(Vmax/mg protein)
0
2
4
6
8
*
#
Young
Aged
Aged-Ex
c.
Young
Aged
Aged-Ex
Tryptase
GAPDH
Tryptase/GAPDH
0
1
2
3
4
5
*
#
Young
Aged
Aged-Ex

Figure 4

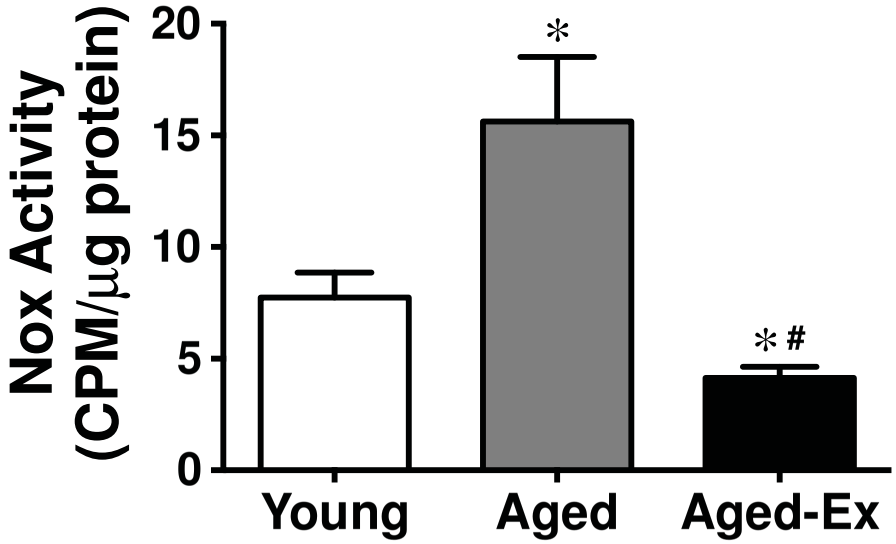

Figure 5

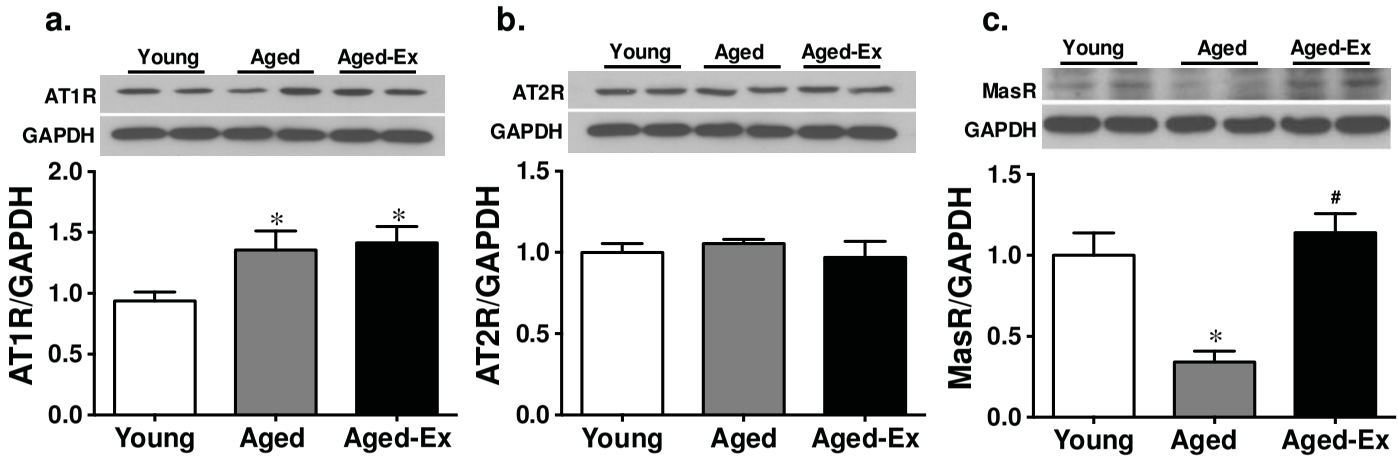
a.
Young
Aged
Aged-Ex
AT1R
GAPDH
AT1R/GAPDH
2.0
1.5
1.0
0.5
0.0
*
*
Young
Aged
Aged-Ex
b.
Young
Aged
Aged-Ex
AT2R
GAPDH
AT2R/GAPDH
1.5
1.0
0.5
0.0
Young
Aged
Aged-Ex
c.
Young
Aged
Aged-Ex
MasR
GAPDH
MasR/GAPDH
1.5
1.0
0.5
0.0
*
#
Young
Aged
Aged-Ex

Figure 6

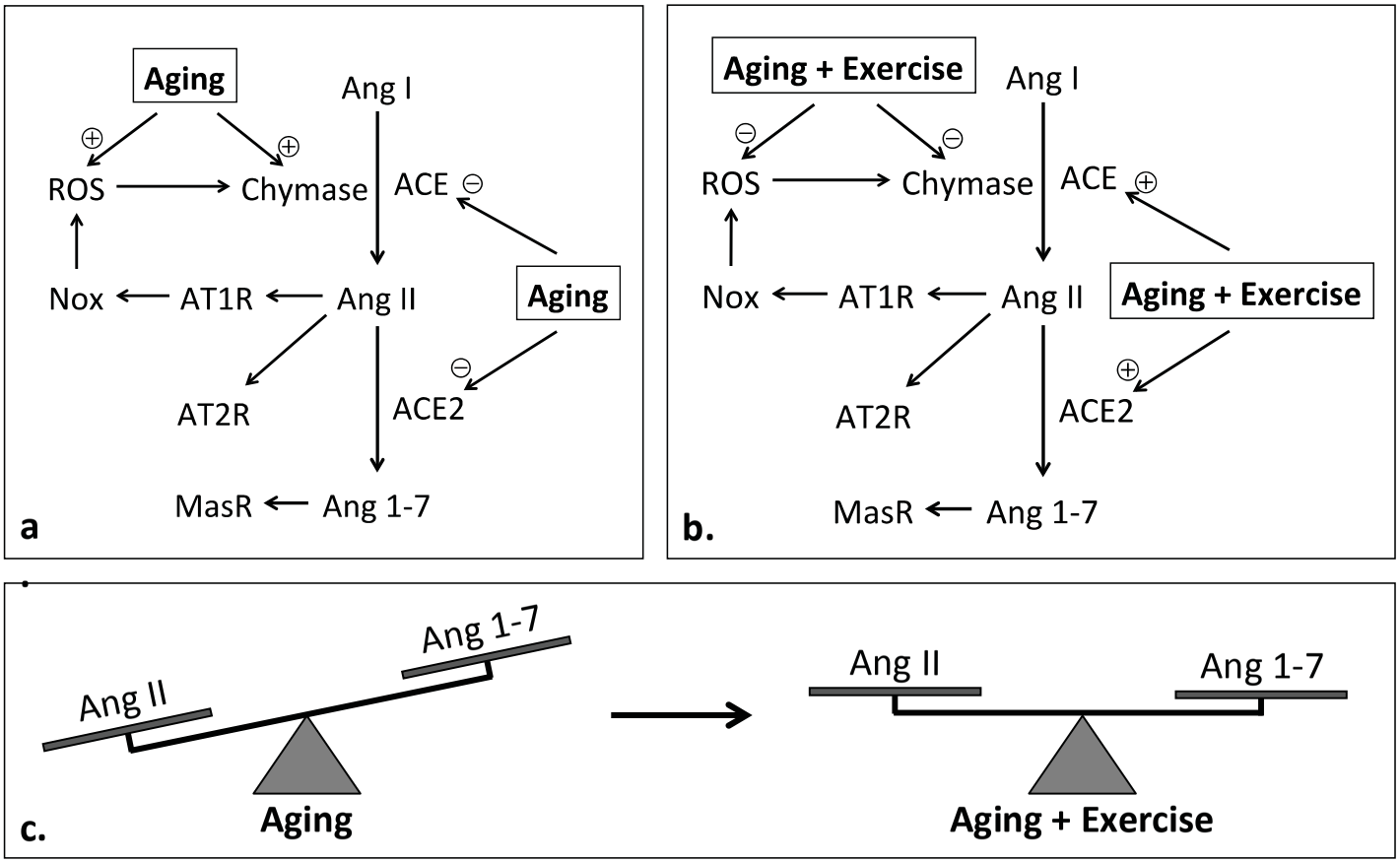
Aging
Ang I
ROS
Chymase
ACE
Nox
AT1R
Ang II
Aging
AT2R
ACE2
MasR
Ang 1-7
a
Aging + Exercise
Ang I
ROS
Chymase
ACE
Nox
AT1R
Ang II
Aging + Exercise
AT2R
ACE2
MasR
Ang 1-7
b.
Ang II
Ang 1-7
Aging
c.
Ang II
Ang 1-7
Aging + Exercise

Figure 7