Exercise training restores IGF1R survival signaling in d-galactose induced-aging rats to suppress cardiac apoptosis

Ing-Shiow Lay a,b, Wei-Wen Kuo c, Marthandam Asokan Shibu d, Tsung-Jung Ho e,f,g, Shiu-Min Cheng h, Cecilia Hsuan Day i, Bo Ban j, Shulin Wang k, Qiaowen Li k,1, Chih-Yang Huang a,d,l,m,n,1,⇑

⇑Corresponding author at: Cardiovascular and Mitochondrial Related Disease Research Center, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien 970, Taiwan.
E-mail address: cyhuang@mail.cmu.edu.tw (C.-Y. Huang).

1 These authors share equal contributions.

Introduction: Insulin-like growth factor-I receptor (IGF1R) mediated survival signaling is a crucial mechanism for cellular endurance and a potential indicator of recuperation in deteriorating hearts.
### Introduction

Aging is a complicated biological process that causes a gradual but steady decline in the normal physiological and biochemical functions. Successful advancements in the health care have somewhat resulted in an increase in the population of the aging society. Lack of physical activity may also associate with aging with corresponding decline in physical function. Aging causes substantial impact on normal health which include disorders such as muscle atrophy [1], Alzheimer’s disease [2], and cardiovascular diseases [3]. Cardiovascular diseases are a major cause of death worldwide, and increase in the global aging population has also led to increase in the mortality due to cardiovascular diseases which is estimated to reach 23.3 million people by 2030 [4].

Exercise training is considered as an efficient strategy in the treatment of cardiovascular diseases [5,6]. Exercise improves the antioxidative capacity and mitochondrial viability in the heart cells and cardio-protection of exercise is known to be correlated with physiological cardiac growth that is distinct from pathological hypertrophy [7,8]. Exercise training in diabetic murine models has shown to provide PGC-1α and Akt mediated cardioprotective effects [7,9]. Mitochondria are responsible to address various physiological or metabolic demands, and they play an important role in cellular proliferation [10]. Various studies have confirmed that the aging disorders are associated with the loss of mitochondrial homeostasis and deterioration of tissue mitochondrial function [11,12]. Imbalance in mitochondrial function leads to increase in cellular apoptotic events [13]. When the mitochondrial membrane potential is lost, the mitochondrial cytochrome c is released to cytosol which subsequently results in cellular apoptosis [14]. Further, circulating insulin-like growth factor I (IGF-1) levels are negatively correlated with cardiovascular risks and is considered as a prognostic indicator in conditions such as ischemic heart disease but most importantly, reduced levels of IGF-1 in aged persons increases the risk for heart failure [15–17]. IGF-1 signaling is transduced by its transmembrane tyrosine kinase receptor IGF1R. Docking of IGF-1 to its receptor results in the activation of its downstream signaling in cardiomyocytes by triggering receptor autophosphorylation [18,19]. The downstream signaling cascades subsequently regulate metabolism, cellular proliferation, differentiation, cellular hypertrophy and cell survival in heart [15]. IGF1R mediated activation of Akt survival pathway and the ERK pathway are well studied in cardiomyocytes for their involvement in cell survival [20,21]. Activated Akt may exhibit direct inhibition effects on pro-apoptotic Bcl-2 family proteins [22,23]. In addition, recent studies show that MitoKATP-mediated mitochondrial translocation of pAkt potentially provide cardio-protection against hypoxia-induced apoptosis [24]. For its importance for being a survival factor, it is imminent to determine if IGF1R activation can be restored in aging condition following exercise.

In the present study, the effects of exercise on D-galactose induced aging associated reduction in IGF1R function and cardiac damages were determined on Sprague-Dawley rats. The results show a positive influence of IGF1R in aging animals.

### Methods and Material

**Animal experiments**

Three weeks old male Sprague-Dawley (SD) rats were procured from BioLASCO (A Charles River Licensee Corporation, Yi-Lan, Taiwan). The rats underwent adaptation for a week. The rats were provided with standard laboratory diet (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and drinking water was provided ad libitum and were properly housed in optimized temperature (24 ± 2 °C and humidity 55 ± 10%). Rats were segregated into different groups (n = 9): Control group, normal rats with saline. The swimming training was performed following previous report by Hart, et al [25]. In the first two weeks, the normal rats from exercise training group and aged rats from the aging group with swimming exercise were left for swimming for 20 min/day, 5 times/week. The duration of swimming was extended to 30 min/day starting from the 3rd week and to 60/min during fourth to eighth week. The rats were left for swimming in 50 cm deep of water maintained at 35 ± 1 °C [26] individually in a 60 x 90 cm water tub. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan. The study followed the principles of laboratory animal care [27].

**Western blotting analysis**

Protein extracts were derived upon homogenization using tissues in a lysis buffer (100 mg tissue/mL buffer) containing 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, deoxycholic acid (0.25%), NP-40 (1%), 1 mM EDTA. After centrifuging the homogenates at
12,000g for 40 min the supernatants were collected and stored at −80 °C. The protein concentration was determined by Lowry method and the Western blotting analysis was performed following methods mentioned in other reports with slight modification [28]. The PVDF membranes with transferred proteins were blocked in 3% bovine serum albumin (BSA) in TBS buffer. The primary antibodies Fas-L(SC-956), FADD(SC-6035), Caspase-8 (SC-6134), Bax (SC-526), α-tubulin(SC-5286), β-actin (SC-47778), p-IGF1R (sc-101703), Akt (SC-5298) and Bcl-2 (SC-7382) were purchased from Santa Cruz Biotechnology (California, USA); IGF1R (ab19675) was from Abcam Biotechnology (Cambridge, UK) and p-Akt (#9275), Cleaved caspase-3 (#9664) and PARP (#9542) were from Cell Signaling technologies (Maryland, USA), Cell Signaling, Maryland, USA). Appropriate secondary antibodies were used and the immunoblots were visualized and documented with Fujifilm LAS-4000 (GE healthcare UK limited, Buckinghamshire, UK).

**Masson’s trichrome staining and TUNEL assay**

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay for the tissue sections was performed as mentioned in the previous report [29]. Briefly, the de-waxed and rehydrated tissue sections were treated with proteinase K followed by permeabilization solution and then with TUNEL reagent (Roche Applied Science, Indianapolis, IN). The nucleus was then stained using 4, 6-diamidino-2-phenylindole (DAPI). The TUNEL-positive nuclei were illuminated in green and DAPI stained nucleus were in blue. Photomicrographs were obtained using fluorescent microscope (DP 74, Olympus, Tokyo, Japan). Masson’s trichrome was performed following procedure mentioned in previous report [30] and the de-waxed and rehydrated tissue sections were placed in freshly prepared Weigert’s Hematoxylin reagent for 10 min and then in Briebrch Scarlet for 10 min followed by placing in phosphotungstic phophomolybdic acid for 10 min. After applying anilin blue solution for 10 min and in 1% glacial acetic acid, the slides were dehydrated with graded ethanol and photomicrographs were captured in Zeiss Axioshot microscope (Zeiss, Oberkochen, Germany).

**Statistical analysis**

The results are shown as means ± SEM obtained from 3 independent experiments. The differences among the groups were analyzed by one-way ANOVA analysis using Graphpad prism software (GraphPad Software Inc, San Diego, CA, USA) followed by Tukey’s post hoc test and all results were quantified using ImageJ software (NIH, MD). p < 0.05 was considered statistically significant.

**Results**

**Exercise training attenuates aging-associated cardiac apoptosis**

TUNEL assay was performed to determine if d-galactose induced-aging could induce cardiac apoptosis and if exercise training could help overcome the aging-associated cardiac apoptosis. TUNEL assay on the left ventricular section from aging-induced rats show that increased number of apoptotic nuclei compared to the control group. The number of TUNEL-positive cardiac cells was 6.5 folds higher in d-galactose induced-aging rats and exercise treatment in the aging rats reduced the apoptosis by 66% with respect to the number of apoptotic cells (Fig. 1).

**Effects of aging and exercise training on cardiac fibrosis**

Masson’s Trichrome staining of the heart sections show that d-galactose induced-aging rats displayed large amounts of collagen accumulation which was reduced in aging rats with swimming

![Fig. 1](image-url)
The results therefore show that exercise training in aging group potentially restore the cardiac contractile function due to reduction in the cardiac remodeling.

Exercise training on D-galactose-induced modulations in cardiac extrinsic apoptotic pathway

To determine the changes in the cardiac Fas receptor-involved apoptosis mechanism in the aging model after exercise training, Fas ligand (Fas-L), FADD and active caspase-8 levels were checked by Western blot analysis. The results reveal that Fas-L and FADD increased significantly in the aging rats (Fig. 3) and thereby revealed the involvement of these factors in the aging-associated apoptosis. Further, while exercise training did not cause any notable changes in the apoptosis events in normal rats, exercise training in the aging group were significantly reduced up on exercise training as evident the active caspase-8 protein levels. Therefore, 8 weeks of exercise training potentially ameliorates aging associated extrinsic apoptosis.

Modulations in aging associated cardiac intrinsic apoptotic pathway

To determine the changes in the mediators of cardiac mitochondria-dependent apoptotic pathways, the levels of Bax, cleaved caspase-3 and PARP were analyzed by Western blotting (Fig. 4). The results show that cardiac Bax, cleaved caspase-3 and PARP levels were significantly elevated in the aging group. Meanwhile, there was no notable change in Bax, cleaved caspase-3 and PARP levels between the control group and the normal rats with exercise training. But, aging rats with exercise training showed a strong reduction in the level of cleaved caspase-3. The results therefore reveal that exercise training effectively suppress aging associated intrinsic apoptosis.

Effect of exercise training on cardiac pro-survival pathway

Further to ascertain the changes in cardiac IGF1R/AKT survival cascade in the aging rats caused due to exercise training, the pro-survival associated proteins of the heart were measured by western blotting (Fig. 5). The cardiac p-IGF1R, p-Akt and Bcl-2 protein levels were significantly downregulated in the aging group. However, exercise training showed significant enhancement in the levels of survival proteins like IGF1R, p-Akt & Bcl-2 in the aging rats. The results therefore highlight that IGF1R associated survival cascade is an important prognostic even in exercise induced protection against the pathological phenomenon of aging.

Discussion

Various alternative approaches have been demonstrated to deliver cardioprotective effects against numerous pathological conditions [30–37]. According to American College of Sports Medicine and the American Heart Association exercise training provide car-
Diabetes protection in conditions like hypertension, coronary artery disease and it is also widely known to attenuate ischemia-reperfusion injury in aged adults [38,39].

In this study, nuclear staining in hearts of β-galactose-induced aging mice showed that there was a 15% increase in the number of apoptotic nuclei in cardiac cells. However, the β-galactose-induced aging mice with exercise training, the cells apoptosis found to be reduced to 3%. Various study show that apoptotic rates of 2–12% affect the physiological function of the heart and cause irreversible damage to the heart and affects systemic blood supply [40].

Our previous study also demonstrated that aging triggers cardiac cell death mediated by Fas-L that binds to its receptor Fas. Fas-L with Fas will activate its death-domain and downstream protein FADD and upon the release of pro-caspase-8 they combine with FADD in the cytoplasm to activate caspase-8 that directs reactivation of caspase-3 protein and induce the apoptosis program. Activation of Caspase-8 could also lead Bid into t-Bid. The t-Bid gets embedded into the mitochondrial outer membrane, causing mitochondrial released of cytochrome-c which induces Caspase-3 activation by Caspase-9 and triggers cell apoptosis. In our results, the Caspase-8 and Fas-FADD were augmented in β-galactose induced aging group and were reduced in exercise training group [41]. The results suggest that β-galactose induced aging rats, similar to natural aging rats, display cardiac physiological changes and increased cardiac cell death (Fig. 6). Suppression of Fas-FADD path-

Fig. 4. Effect of exercise training on proteins of intrinsic apoptosis. (A) Representative protein products of Bax, Cleaved Caspase-3 and PARP from left ventricles of Control (C), exercise training (E), aging (A) and aging with exercise (AE) group rats were measured by Western blotting analysis. The β-actin was used as an internal control. (C) Bars represent the relative fold changes in protein levels representing mean values ± SEM. *P < 0.05, and ***P < 0.001 represent significant differences with respect to control group. ###P < 0.001 represents significant differences with respect to aging group.

Fig. 5. Exercise reboots survival signaling in the cardiac cells of aging rats (A) representative protein products of IGF1R, p-IGF1R, Akt, p-Akt and Bcl-2 extracted from the left ventricles of Control (C), exercise training (E), aging (A) and aging with exercise (AE) group rat hearts were measured by Western blotting analysis. The β-actin was used as an internal control. (C) Bars represent the relative fold changes in protein levels representing mean values ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 significant differences with respect to control group. ##P < 0.01 and ###P < 0.001 significant differences with respect to aging group.
way by exercise training is a potential way for controlling myocardial cellular apoptosis during the progression of cardiac disease. In line with other reports, our results show that α-galactose induced aging causes a notable increase in cleaved Caspase-3 levels and PARP cleavage [42,43]. It should be also noted that, Caspase-3 and PARP remain inactive in control rats and in normal rats with exercise.

The PI3K-Akt signal pathway is a notable survival mechanism that could be regulated by IGF1R. Enhancement of IGFIR and its associated survival factors is considered as a hallmark of efficient cardioprotection against various pathological models of cardiac defects [30–32,35,37,44]. IGF-I and its associated pro-survival proteins p-PI3k, p-Akt, Bcl-2 and Bcl-xl were seen to be elevated in STZ induced diabetic models as well, indicating their compensatory survival mechanism to suppress apoptosis under various stresses [15,29,45]. In our study, p-Akt/Akt were significantly elevated in young mice with exercise training and given exercise training after α-galactose-induced aging mice. These results suggested that regardless of young or old mice, PI3K-Akt signal cascade plays a central role in cellular survival mechanism and exercise training is an effective means in maintaining the PI3K-Akt activation in vivo. Further, IGF1R survival mechanism could be a possible hallmark for the beneficial effects in heart conferred upon exercise training.

**Funding**

This study is supported in part by Asia University, Taiwan and China Medical University, Taiwan (CMU103-ASIA-17).

**Author contributions**

Ing-Shiow Lay and Chih-Yang Huang designed the study. Chih-Yang Huang, Tsung-Jung Ho and Cecilia Hsuan Day verified the data. Marthandam Asokan Shibu drafted the manuscript. Wei-Wen Kuo performed statistical analysis. Shiu-Min Cheng, Bo Ban, Shulin Wang, Qiaowen Li proof read the manuscript. Chih-Yang Huang obtained funding and provided resources for the study.

**Compliance with Ethics Requirements**

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgment**

The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation, and the results of the present study do not constitute endorsement by ACSM.

---

Fig. 6. Schematic representation on the molecular events involved in cardio-protection provided by exercise training.
