Compensatory effect of IGF-I survival signaling is reduced by aging to accelerate apoptosis in cardiac cells exposed to second-hand smoke

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Introduction: Secondhand smoke (SHS) exposure is associated with an increased risk of coronary artery disease. Ageing is a physiological process involving progressive impairment of normal heart functios, due to an increasing vulnerability, which reduces the ability of survive. The aim of this study was to examine secondhand smoke exposure in aging-related death-survival balance. **Methods:**Rats were divided into two age groups, male young adult and male old which were divided into two subgroups and treated for 4 weeks SHS exposure as follows: Control (C), not exposed to SHS. Secondhand smoke exposure (S), exposed to SHS. The rats were placed in exposure chamber and exposed to 10 cigarettes for 30 min, twice a day, five days/week for 1 month. Then, rats underwent morphological and function study with echocardiography. Histopathologic of left ventricular sections were stained with hematoxylin-eosin staining and related death-survival protein expression by Western Blotting. **Results:**From echocardiography results, we found EF (%) and FS (%) were apparently decreased in young and aging rat under SHS two weeks exposure, and more severe in aging but compensated occurred in young rats after four weeks exposure. LVID, LVPW and IVS at systolic diameters were increased in young SHS-exposed rats, and enhanced more in aging SHS exposed rats. In addition, both upregulation of death receptor dependent apoptosis pathways (TNFα/Fas-L-Fas/FADD-cleaved caspase 8) in young and aging SHS exposure animals, and more enhanced in aging SHS exposed rats. However, the Mitochondria apoptosis proteins, t-Bid, Bid, cytochrome *c* and the ratio of Bad/Bcl 2 were only increased in aging and augumented increased in aging SHS exposure rats. Similarly results were observed on cleaved-caspase 9 and 3 proteins. Moreover, the survival pathways (IGF-I/IGFIR-PI3K/p-Akt) was found only compensated in young SHS exposure, but not in aging rats and even more decreased in aging SHS exposed rats. **Conclusion:** Aging and SHS should be considered as risk factors for cardiac dysfunction. Moreover, Aging reduces the IGF-I compensated signaling and accelerate the cardiac apoptotic effects induced by Second-hand Smoke.

Materials and Methods

Animals

We purchased male Hamster rats (young age group: 6 weeks of age; body weight, 132.5 ± 4.61 g; old age group: 18 months of age, body weight, 145 ± 3.42 g) from the National Science Council Animal Center, Taipei, Taiwan. The animals were housed six in individual cages in an environmentally controlled animal room, and tap water was provided . All animals were handled according to the guidelines of the Taiwan Society for Laboratory Animals Sciences for the care and use of laboratory animals in temperature- and humidity-controlled chambers.

Experimental groups and secondhand smoke (SHS) exposure

Rats were divided into 2 age groups, young adult and old, which were each divided into two subgroups and exposed to SHS for 4 weeks as follows: 1) control (C), 6 animals not exposed to cigarette secondhand smoke, and 2) secondhand smokers (S), 6 animals exposed to cigarette secondhand smoke (SHS). The rats were placed in whole-body exposure chambers and exposed to 10 cigarettes. Filtered air was introduced into the chamber at a low rate. Rats were exposed to cigarette smoke for 30 min, twice a day for five days per week for 1 month. Room temperature was maintained at 22–25°C, and relative humidity was approximately 40%.

Western blotting

We prepared the tissue extracts as described above. SDS-PAGE was carried out with polyacrylamide gels. The samples were electrophoresed at 100 V for 1 hr. Electrophoresed proteins were transferred to nitrocellulose paper using a Hoefer Scientific Instruments Transphor unit at 100 mA for 14 hr. We incubated nitrocellulose papers in blocking buffer for 2 hr at room temperature. Monoclonal antibodies were diluted 1:200 in antibody binding buffer (TBS). Incubations were performed at room temperature for 3.5 hr. We washed the immunoblots three times in 50 mL of blotting buffer for 10 min. The membranes were then immersed in the second antibody solution containing alkaline phosphatase goat anti-rabbit IgG diluted 1,000-fold in binding buffer for 1 hr. The membranes were then washed in blotting buffer for 10 min three times. Color development was performed by ECL chemiluminescence.

Statistical analysis

All data are expressed as the mean±standard error of the mean (SEM). For western blot analysis, quantitation was carried out by scanning and analyzing the intensity of the hybridization signals using the FUJIFILM Imagine program. Statistical analysis of the data was performed using SigmaStat software. Comparison between groups was conducted using a two-way analysis of variance (ANOVA). p values of less than 0.05 and 0.01 were considered to be statistically significant and highly statistically significant, respectively.

Results

Echocardiography demonstrated that left ventricular function decreased in young and aging rats under a two-week SHS exposure and this decrease was more severe in aging rats, while young rats exhibited compensation after four weeks of exposure.

	MYC(3)	MYS(3)	MOC(3)	MOS(3)
LVIDd (baseline, mm)	4.3±0.5	3.4±0.3	4.0±0.3	4.0±0.07
LVIDd (2 weeks, mm)	3.2±0.2	3.7±0.9	3.3±0.3	3.3±0.03
LVIDd (4 weeks, mm)	2.6±0.3	4.3±0.3*	3.7±0.4	4.3±0.03*
LVIDs (baseline, mm)	2.6±0.4	2.1±0.3	2.6±0.2	2.3±0.06
LVIDs (2 weeks, mm)	1.8±0.1	2.3±0.9	2.5±0.3	2.3±0.03
LVIDs (4 weeks, mm)	1.8±0.1	3.0±0.0 **	2.5±0.3 *	3.3±0.03 *
LVPWd (baseline, mm)	1.2±0.2	1.2±0.2	1.2±0.1	1.2±0.00
LVPWd (2 weeks, mm)	1.0±0.0	1.0±0.0	1.0±0.1	1.3±0.03
LVPWd (4 weeks, mm)	1.0±0.0	1.7±0.7	1.0±0.0	1.0±0.00
LVPWs (baseline, mm)	1.9±0.1	1.7±0.7	1.8±0.1	1.9±0.03
LVPWs (2 weeks, mm)	1.8±0.1	1.7±0.3	1.7±0.0	2.0±0.00 *##
LVPWs (4 weeks, mm)	1.7±0.1	2.0±0.0*	1.7±0.0	2.0±0.00 *##
IVSd (baseline, mm)	1.3±0.1	1.2±0.1	1.0±0.1	1.4±0.01
IVSd (2 weeks, mm)	1.0±0.0	1.0±0.0	1.0±0.0	1.3±0.03
IVSd (4 weeks, mm)	1.1±0.1	1.3±0.3	1.0±0.0	1.3±0.03
IVSs (baseline, mm)	1.8±0.1	1.9±0.2	1.7±0.0	2.1±0.02
IVSs (2 weeks, mm)	1.6±0.2	2.0±0.0	1.7±0.0	2.0±0.00
IVSs (4 weeks, mm)	1.6±0.2	2.0±0.0*	1.7±0.0	2.0±0.00*##

Table 1. Left ventricular function

	MYC(n=3)	MYS(n=3)	MOC(n=3)	MOS(n=3)
EF (baseline, %)	77.05±5.48	72.91±3.47	59.88±8.03	69.47±6.26
EF (2 weeks, %)	81.33±1.20	66.33±7.75	75.12±2.82	63.33±2.67*##
EF (4 weeks, %)	84.33±1.84	73.33±3.18*	75.30±2.66*	56.33±8.57*
FS (baseline, %)	40.99±5.46	36.51±2.85	28.15±5.50	34.49±4.71
FS (2 weeks, %)	47.28±2.28	32.33±5.24*	31.90±1.64**	29.33±1.67**
FS (4 weeks, %)	47.28±2.28	37.00±2.52*	38.75±2.27	25.67±4.67*

*MYC VS. MYS, MOC, *P<0.05, **P<0.01, # MOC VS. MOS, # P<0.05, # # P<0.01

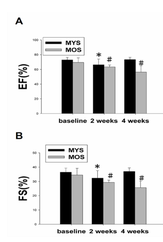

Fig 1. Representative echocardiography of cardiac function
A. Fractional shorting (FS%). B. Ejection fraction (EF%). FS% and EF% were decreased in response to secondhand smoke (SHS) exposure in aging rats after 2 and 4 weeks of exposure. However, in young age rats, exposure to SHS for 2 weeks caused a decrease in cardiac function, but after 4 weeks of SHS exposure, function was increased. *p<0.05 vs baseline in young age, #p<0.05 vs baseline in old age.

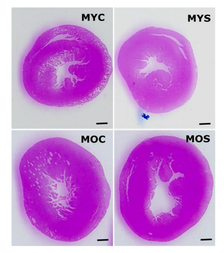

Fig 2. Morphological features of the left ventricle by hematoxylin-eosin staining
Histological analysis of cardiac tissue sections stained with H&E. Increased left ventricular wall thickness and decreased left ventricular chamber were observed in young rats exposed to SHS (MYS), old control rats (MOC), and old rats exposed to SHS (MOS).

Upregulation of death receptor dependent apoptosis pathways (TNFα/Fas-L-Fas/FADD-cleaved caspase 8) occurs in young and aging SHS-exposed animals and was enhanced in aging SHS-exposed rats.

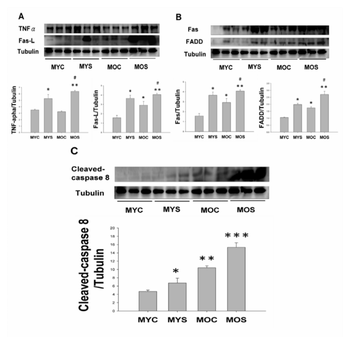

Fig 3. Death receptor-dependent signaling pathway (Fas-L/Fas) in young and aging rats with or without SHS exposure.
A. Western blotting of the activated form of myocardial TNF α and Fas-L from the left ventricle. TNF α and Fas-L were increased in SHS exposed rats in both young and old age groups. B. The downstream Fas and FADD were increased similar to TNF α or Fas-L. α-tubulin was used as a loading control. *p<0.05, **p<0.01 *versus* male young controls (MYC), #p<0.05, ##p<0.01 *versus* male old controls (MOC). C. Cleaved caspase 8 was progressively increased in young rats exposed to SHS (MYS), old control rats (MOC), and old rats exposed to SHS (MOS). *p<0.05, **p<0.01, ***p<0.001 *versus* young male controls (MYC).

Mitochondria apoptosis proteins were only increased in aging rats, and the increase was augmented in aging SHS-exposed rats.

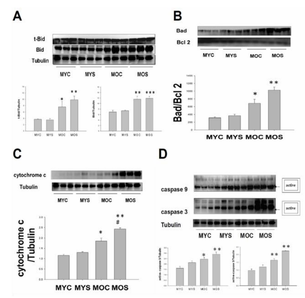

Fig 4 . Mitochondrial-dependent apoptosis signaling pathways in young and aging rats with or without SHS exposure.
Quantification of densitometry analysis of protein levels. *p<0.05, **p<0.01 significant differences compared with the male young controls. A. Bid and t-Bid were increased in male old controls (MOC) and male old rats exposed to SHS (MOS), but not in male young aged rats; *p<0.05, **p<0.01 versus male young controls (MYC). B. The ratio of Bad and Bcl2 was increased in male old controls and male old rats exposed to SHS, but not in male young rats; *p<0.05, **p<0.01 versus male young controls (MYC). Cytochrome c released from mitochondrial was increased in old male controls and old male rats exposed to SHS; *p<0.05, **p<0.01 versus male young controls (MYC). Aging rats exposure to SHS was higher compared with male old controls (MYC); #p<0.05 *versus* male old controls (MOC). D. Cleaved caspase 9 was increased in male old controls (MOC) and old rats exposed to SHS (MOS); *p<0.05, **p<0.01, *versus* male young controls (MYC). Cleaved caspase 3 was also increased in male old controls (MOC) and old rats exposed to SHS (MOS); **p<0.01, *versus* male young controls (MYC).

The cardiac survival pathway IGF-I/IGFIR-PI3K/p-Akt was found to be attenuated in young SHS exposed rats, but not in aging rats; especially those aging rats exposed to SHS.

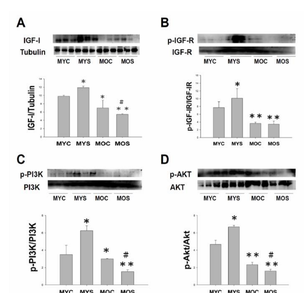

Fig 5. Suppression of the cell survival signaling pathway IGF-I/IGF-IR/PI3K/AKT was observed in aging and in aging plus exposure to SHS rats, but compensatory effects were observed in young SHS-exposed rats.
A. Circulating IGF-I was increased in male young rats exposed to SHS (MYS) compared to male young control (MYC). In male old rats and aging rats, exposure to SHS decreased circulating IGF-I; *p<0.05, **p<0.01 versus male young control (MYC). B. The ratio of p-IGF-IR/IGF-IR was increased in male young rats exposed to SHS (MYS) compared to male young controls (MYC) and was decreased in old control rats and aging rats exposed to SHS; **p<0.01 versus male young control (MYC). C. The ratio of p-PI3K/PI3K was increased in young rats exposed to SHS (MYS) (p<0.05) and decreased in old control rats (MOC) and aging rats exposed to SHS (MOS); *p<0.05, **p<0.01 versus male young control (MYC). The p-PI3K/PI3K ratio in male old rats exposed to SHS was higher compared to male old controls (MOC); #p<0.05 *versus* male old control (MOC). D. The ratio of p-AKT/AKT was the same as the p-PI3K/PI3K increase in male young rats exposed to SHS; *p<0.05 versus male young controls (MYC). However, it was decreased in male old controls (MOC) and male old rats exposed to SHS (MOS); **p<0.01 versus male young controls (MYC). The ratio in male old rats exposed to SHS was higher compared with male old controls (MOC); #p<0.05 *versus* male old control (MOC).

Discussion

We believe SHS and aging both enhanced left ventricular hypertrophy. These results indicate that SHS exposure and aging induce upregulation of mitochondria-dependent and –independent apoptosis signaling pathways and downregulation of survival signaling pathways. Moreover, aging reduces IGF-I compensated signaling and accelerates the cardiac apoptotic effects induced by second-hand smoke. Aging and SHS should both be considered as risk factors for cardiac dysfunction.

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