Activation of IGF-I Survival Signaling and Its Compensative Inhibition of the Cardiac Apoptosis on Carotid Arteries Balloon-Injured Rat Hearts

Cheng-Hong Hsieh1, #, Peiying Pai2, #, Jia-Ping Wu3, Tsung-Jung Ho4, 5, Chieh-Hsi Wu6, Marthandam Asokan Shibu3, Cecilia Hsuan Day7, Vijaya Padma Viswanadha8, Ho-Lin Chuang9, 10, 11, and Chih-Yang Huang1, 3, 4

1Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan
2Division of Cardiology, China Medical University Hospital, Taichung 40402, Taiwan
3Graduate Institute of Basic Medical Science, China Medical University, Taichung 40402, Taiwan
4Graduate Institute of Chinese Medicine, China Medical University, Taichung 40402, Taiwan
5Chinese Medicine Department, China Medical University Beigang Hospital, Yunlin 63244, Taiwan
6Graduate Institute of Pharmacology, China Medical University, Taichung 40402, Taiwan
7Department of Nursing, MeiHo University, Pingtung 91202, Taiwan
8Department of Biotechnology, Bharathiar University, Coimbatore 641046, India
9Shanghai Qunyou Trading Co., Ltd. SHANGHAI, 200335, China.
10Zen Transmission Foundation of Medical Culture and Education, Taichung 43642, Taiwan
11Taiwan Chinese Medical Association, Taichung 43642, Taiwan

Abstract

In this study, a rat carotid balloon injury-animal model was used to elucidate the temporal relation of hypertrophy in the progression of cardiac damage and the role of insulin-like growth factor (IGF)-I survival pathway on course of the cardiac damage. Rats were subjected to carotid balloon-injury and examined at different time points. We further studied the heart-weight/body-weight-ratio, histology and protein expression to understand the pathological events associated with percutaneous transluminal coronary angioplasty (PTCA) induced damages. Protein expression analysis showed increased levels of IGF-I signaling pathway and mitogen-activated protein kinase (MAPK) signaling pathway after 2 h and after 2 d of carotid balloon injury. On the other hand, apoptosis signaling pathways were enhanced after 14 d of carotid balloon injury. According to the results, rat carotid balloon injury significantly induced IGF-I survival signaling and compensated hypertrophy pathway during the initial period of injury however after 14 d the proteins involved in apoptotic cell death were elevated and the proteins of the survival pathway and compensatory hypertrophy were significantly reduced.

Key Words: cardiac apoptosis, compensated hypertrophy, decompensated hypertrophy, IGF-I survival pathway, percutaneous transluminal coronary angioplasty
**Introduction**

Blood vessel injury has been long studied on various experimental animal models to determine the consequences of injury as an effort to interpret these findings to human. Percutaneous transluminal coronary angioplasty (PTCA) procedure in artery is known to inflict damages to the artery leading to inflammation and hypertrophic conditions (17). PTCA has been used to patients who were treated with prior coronary artery bypass grafting after native coronary artery stenosis (11, 13) and is a useful alternative to coronary artery bypass surgery and is performed to rectify symptomatic vessel coronary artery disease (23). PTCA is also an innovative treatment for proximal short-segmental, non-calcified, concentric isolated coronary stenosis with coronary artery disease.

Atherosclerosis produces scattered areas of blockage within a coronary artery. PTCA is a technique used to dilate an area of arterial blockage with catheter that has an inflatable small sausage-shaped balloon at the tip. It has been used at appropriate frequency in patients of symptomatic coronary artery disease, including multi-vessel disease unstable angina, acute myocardial infarction and total occluded coronary arteries (25). The survival rate after a successful PTCA is 93% and the possibility of restenosis occurs in 30–50% of cases within six months after successful PTCA.

But PTCA is often followed by vascular injury and often results in reocclusion of the artery or restenosis across the internal elastic lamina to form a neointima. A coronary restenosis also leads to 100% occlusion during angioplasty. Reports show that 30%–50% of balloon dilation ends in restenosis within six months. It may in selected cases also dramatically improve myocardial blood supply (1). Neointima occlude the artery induced compensated stage hypertrophy to decompensated stage dilation hypertrophy that progress to congestive heart failure (CHF) after PTCA (9).

Cardiomyocyte hypertrophy (compensated stage associated with normal cardiac function, and increased in left ventricle (LV) wall thickness) is the cellular response to increasing LV wall tension with enhanced protein synthesis (4). Similarly stress associated with PTCA invokes hypertrophy as a cardiac response. Although, hypertrophy is an adaptive response to a pathological stress, their positive effects are limited to “compensated hypertrophy”. Whereas, hypertrophy following PTCA associated carotid artery balloon injury causes dilation of cardiomyocytes; damage to heart muscle; thinning of LV wall and dilation of cardiac chambers. Under such stressful conditions compensatory effects of insulin-like growth factor (IGF)-I signaling pathway and cell death associated Fas-L signaling pathway are often known to mediate molecular mechanisms responsible to regulate cardiomyocyte proliferation and recovery. Meanwhile, patients with cardiac hypertrophy often exhibit myocyte necrosis followed by fibrosis in the LV. Myocardial fibrosis may contribute to increased ventricular chamber stiffness and impaired relaxation, at least in part from wall thinning and scar formation, causing transition to heart failure.

IGF-1 which has growth-promoting actions on a variety of tissues including the heart is involved in mediating physiological and pathological adaptations during cardiac hypertrophy (16, 18, 19, 24). Circulating IGFs in plasma mostly are bound to their specific IGF-binding proteins (IGFBPs) that act as carrier protein. IGFBPs serve not only to transport IGFs in the circulation but also to prolong the half-lives and modulate the tissue specificity of IGFs. Receptors for IGF-I include both IGF-I and IGF-II receptors however, IGF-I profoundly bind to IGF-I receptor than to IGF-II receptor. Enhancement of IGFBP-3 is known to inhibit IGF-I-induced cardiac hypertrophy (12, 16, 18). IGF-I reduces apoptosis and relies exclusively on the phosphinositide 3 kinase (PI3K)/Akt pathway activation and PI3K activation increases cell survival and antagonizes apoptosis after PTCA. Akt activity is essential for both basal cell growth and adaptive (physiological) and maladaptive (pathological) LV hypertrophy. In this work we investigated whether IGF-I signaling pathway and associated molecular event are induced in response to biochemical stress accumulated due to PTCA (16). A rat carotid artery balloon injury model was used to determine the modulation in the survival signaling and apoptosis mechanism at different time intervals (2 h, 2 d and 14 d). The results reveal that IGF-I regulates cell proliferation and differentiation through IGF-IR after carotid balloon injury. Furthermore, we observed enhancement of both mitochondria dependent and mitochondria independent apoptosis signaling after 14 d of carotid balloon injury (10).

**Materials and Methods**

**Animal Model and Experiments**

All protocols were reviewed and approved by the Institutional Review Board (IRB), Animal care and use committee of the China Medical University, Taichung, Republic of China, and the study was conducted in accordance with the principles of laboratory animal care (15). Male Wistar rats each weighing 300 g were obtained from the Animal House of National Science Council, Taiwan, and housed under 22 ± 5°C, 60 ± 5% relative humidity and 12 h light/dark cycle with
free access to standard pellet feed and tap water. Endothelial denudation and vascular injury was performed in the left common carotid artery of male Wistar rats following previous reports (13, 17, 26). Animals were euthanized after 2 h, 2 d and 14 d.

Histology

Histological analysis was performed according to the methods mentioned in previous reports with slight modifications (6). The rat hearts were perfused and fixed with 10% formalin before embedding in paraffin, all hearts were embedded in a cross section orientation, and all slices were cross sections of the LV. All slices were taken from the LV and fixed in 4% paraformaldehyde and embedded in paraffin. The sections were deparaffinized, rehydrated and stained with hematoxylin-eosin (H&E) staining.

Western Blotting

Western blot analysis was performed by methods suggested in previous reports with minor modifications (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 9.5% polyacrylamide gels (2). After mixing aliquots of cardiac tissue extracts (20 μg) with a suitable volume of phosphate buffered saline (PBS) and 4 μL of 5x bromphenol-blue dye, the mixture was heated at 95°C for 10 min and then rapidly placed in an ice bath. The mixture was spun down in a centrifuge and loaded in polyacrylamide gels and electrophoresed at 110 V for 90 min. Proteins were then transferred onto nitrocellulose membrane (Amersham, Hybond-C Extra Supported, 0.45 Micro) at 150 mA for 2 h. The nitrocellulose membranes were incubated at room temperature for 2 h in a blocking buffer and then in appropriate antibodies against IGF-I (1:250), IGF-IR (1:125), PI3K (1:1000), p-PI3K (1:1000), protein kinase B α (PKβα)/Akt (1:400), p-Akt (1:4000), Bad (1:1000), Bel-2 (1:500), p-MEK 1 (1:1000), MEK 1 (1:1000), extracellular signal-regulated kinase (ERK) 1 (1:1000), p-ERK 1 (1:1000) and α-tubulin (1:500) for 3.5 h. Immunoblots were washed and then immersed in the specific horseradish peroxidase-labelled secondary antibody (Promega) for 1 h. The chemiluminescence visualized after using enhanced chemiluminescence (ECL) was detected and documented using a Fujifilm LAS-3000 (GE Healthcare Life Sciences).

Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). Inter group comparison were performed using one-way analysis of variance (ANOVA) followed by Dunnet's test. P value of <0.05 was considered statistically significant, and P value of <0.001 was considered highly statistically significant.

Results

Histological Assessment and Evaluation of Cardiac Function

The whole hearts from the rats were removed by surgery at various time points (2 h, 2 d and 14 d) after carotid balloon injury. The whole heart weight (WHW) and left ventricular weight (LVW) were found to be increased in the respective rat models after 2 h, 2 d and 14 d of carotid balloon injury (Table 1). The WHW and LVW significantly increased after 14 d of carotid balloon injury. On the other hand, the cardiac histological assessment by hematoxylin and eosin staining show that the size of the LV cells increased after 2 h, 2 d and 14 d of carotid balloon injury (Fig. 1). Carotid balloon injury therefore causes cardiac hypertrophy in rat hearts that may further progress to heart failure. The arrangement of the
cardiomyocytes was also found to be abnormal in the rat hearts after injury. The normal cardiomyocytes in the control rats were tightly packed however, the shape and the arrangement of cardiomyocytes were irregular after 14 d of carotid balloon injury (Fig. 1).
Up-Regulation of IGF-I Survival Signaling Pathway and Down-Regulation of Cell Death Signaling Pathway

The survival signaling pathway that involve IGF-I, IGF-IR, PI3K and Akt was enhanced in the rat hearts after carotid balloon injury. IGF-I and IGF-IR protein expressions increased after 2 h and 2 d after PTCA by western blotting (Fig. 2). The ratio of p-PI3K/PI3K and p-Akt/Akt also increased after 2 h and 2 d (Fig. 3). But after 14 d of carotid balloon injury, the ratio of p-PI3K/PI3K protein expression decreased. Furthermore, the proteins of the intracellular protein kinase signal cascades of hypertrophy, p-MEK1 and ERK1, were analyzed (Fig. 4). In the early period after carotid balloon injury the levels of p-MEK1 and p-ERK1 increased which resulted in the promotion of cardiac compensatory hypertrophy to protect the heart function. However their expressions were significantly down-regulated 14 d after carotid balloon injury. The proteins of the apoptosis pathway such as Fas-L, Fas, Fas-associated protein with death domain (FADD) and Caspase 3 however were low at 2 d after carotid

Fig. 3. The effect of carotid balloon injury on p-PI3K, PI3K, p-Akt and Akt protein expression at 2 h, 2 d and 14 d after injury. Representative western blots showing modulation in the pro-survival PI3K and Akt proteins in rat hearts at 2 h, 2 d and 14 d after carotid balloon injury (*P < 0.05 vs. control, ##P < 0.01 vs. 2 h after surgery).

Fig. 4. MEK (mitogen-activated protein kinase, MAPK kinase) and ERK (MAPK) protein expression level at 2 h, 2 d and 14 d after carotid balloon injury. Western blots showing p-MEK1 (A) and p-ERK1 (B) levels in rat hearts after 2 h, 2 d and 14 d of carotid balloon injury ***P < 0.001 vs. control, ###P < 0.001 vs. 2 h after surgery).
IGF-I Survival Signaling in PTCA-Injured Rat Hearts

balloon injury however the respective protein levels increased after 14 d (Fig. 5). The results indicate that, survival signal pathway strongly suppresses the hypertrophic effect in a compensatory manner and down-regulates cell death in the initial 2 d after carotid balloon injury however 14 d after carotid balloon injury the suppressive effect may become weak due to down-regulation in the survival signaling.

**Effect of Carotid Balloon Injury Apoptosis**

In order to determine the extrinsic apoptosis triggered in response to PTCA the protein expression of Fas-L, Fas and FADD were determined. The level of Fas-L, Fas and FADD decreased after 2 d of carotid balloon injury however the expression of the apoptosis proteins increased after 14 d of carotid balloon injury. Further, mitochondria dependent apoptosis was also analyzed by quantifying the protein expression of bad and Bcl-2 in the cytosol (Fig. 5). Bcl-2 protein expression increased 2 d after carotid balloon injury. Meanwhile, the Bad protein expression decreased after 2 d of carotid balloon injury. However, the level of Bcl-2 decreased and that of Bad increased after 14 d of carotid balloon injury.

**Discussion**

The techniques in PTCA are rapidly evolving to reduce the time constraints and reduce the morbidity. Rat carotid artery balloon injury model has been widely considered in investigating critical morphological, biochemical, molecular and cellular aspects and responses induced by arterial injury. Damage inflicted by balloon catheter destroys the inner endothelial lining causing injury to the vessel and responses such as mitogenesis, migration and apoptosis of smooth muscle cell and causes invasive neointima.
in a time-dependent manner. Therefore carotid artery balloon injury rat model is a valuable tool to investigate various pathophysiological processes in injured vessels (25). In this work rat carotid artery balloon injury model was used to investigate probable reasons behind high mortality and morbidity after PTCA. During PTCA, The balloon catheter is introduced through the skin of the groin, and sometimes the arm is placed within a blood vessel during procedures on coronary arteries (22). Balloon catheters entrap into the lumen of a coronary artery and may encounter trouble due to inadvertent balloon rupture causing rupture of the blood vessel.

Carotid balloon injury is often caused due to removal of endothelial cells and due to stretch-induced damage to medial smooth muscle cells (27). The degree of balloon inflation also determines the extent of vascular injury and subsequent cellular and molecular events associated with neointima development (8). In this study, rat carotid artery balloon injury animal model was used to investigate the time dependent effect at 2 h, 2 d and 14 d after carotid balloon injury. The WHW (g), LVW (g) and histology results revealed the development of hypertrophy in the rat hearts after carotid balloon injury which is a prominent factor for morbidity and mortality (5, 14). WHW (g) and LVW (g) were both increased at 2 h, 2 d and 14 d. Furthermore, the mechanisms of IGF-I survival signaling pathway and apoptosis signaling pathway were investigated by western blotting. Survival signaling pathway and apoptosis signal pathway has been known to be modulated after PTCA (20). In this study, we found that survival signaling pathway suppressed the apoptosis signaling pathway immediately after carotid balloon injury. Further, the downstream IGF-I signaling pathway proteins including IGF-I, IGF-IR, Akt, p-Akt, PI3K and p-PI3K were also found to be increased after 2 d of carotid balloon injury; but the apoptosis signaling pathway proteins including Fas-L, Fas, FADD and caspase 3 were suppressed as determined by western blotting. However, 14 d after injury the control of the apoptosis signaling pathway was deregulated.

On evaluating the extracellular signaling cascade such as MEK1 and ERK1, we observed that the p-MEK1 and p-ERK1 protein levels decreased 14 d after carotid balloon injury and suppressed the compensated cardiac hypertrophy (3, 21). Continued elevation in the activation of MEK1/ERK1 signaling pathway suppressed IGF-I survival signaling pathway related recovery and elevated cell death and induced cardiac hypertrophy after longer period (14 d). At the same time, apoptosis-independent mitochondria signaling pathway proteins including Fas-L, Fas, FADD and active-caspase 3 were also up-regulated and enhanced cardiomyocytes apoptosis. This forms the reason for the-early stage decompensated hypertrophy or the early stage heart failure. We further investigated the mitochondria protein Bel-2 and Bad in the cytosol. According to results, Bel-2 level was elevated and Bad levels were reduced at 2 h and at 2 d time point when compared to the control after carotid balloon injury. Bel-2 protein expression in the cytosol was also decreased at 14 d after carotid balloon injury. However, bad protein expression in the cytosol remained low.

From the results obtained, we conclude that enhanced cardiac compensated hypertrophy occurs at the initial stage after carotid balloon injury and the initiation of decompensated cardiac hypertrophy at 14 d after injury is probably depended on the modulation of IGF-I survival signaling pathway and involves the down-regulation of apoptosis signaling pathways.

**Conflict of Interest**

The authors declared no conflicts of interest.

**Acknowledgments**

This study is supported by Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW105-TDU-B-212-133019) and in part granted by China Medical University (CMU100-Asia-6).

**References**


