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生物科技系碩士班

碩士學位論文

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高密度脂蛋白抑制脂毒性和缺氧對心肌細胞造成的損傷機制探討

High density lipoprotein attenunate lipoptoxity and hypoxia-induced cardiomyocyte damage

指 導 教 授 ：郭薇雯 教授

研 究 生：江麗怡

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致謝

兩年的碩士生活終於結束了，回顧一切真的是酸、甜、苦、辣都有，回想起這兩年辛苦的日子相信一定會覺得這是值得的。在此要感謝口試委員郭志宏老師、劉詩平老師、林靜瑩老師、黃志揚老師以及郭薇雯老師，對我的論文提供了一些不同角度的見解、想法和建議。要特別感謝歐秀中老師和郭薇雯老師很耐心的幫我批改我的論文以及實驗上的教導，以及黃志揚老師在每次的報告都給予我方向使我的研究之路更為順利。

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第一部分

高密度脂蛋白保護棕櫚酸對心肌細胞造成的脂毒性及氧化壓力

Part I

Protective effect of HDL on palmitic acid-induced lipotoxicity and oxidative dysfunction in H9c2 cardiomyoblast cells

中文摘要

過多的飽和脂肪酸與糖尿病,肥胖以及高血脂具有相關性。在心臟中,過多的飽和脂肪酸累積對於造成心衰竭及糖尿病引起的心臟病變扮演重要的角色。有文獻指出,高密度脂蛋白具有高度的生物活性,其中包括膽固醇的流出,抗氧化,抗發炎等功能。而其中還有許多機制尚未釐清,所以本實驗的目的是利用棕櫚酸來模擬一個高脂的環境,來觀察是否高密度脂蛋白可以調控棕櫚酸對心肌細胞造成的損傷及作用機制。我們採用了心臟衍生的 H9c2 肌原母細胞以及新生鼠初代培養的心室細胞,先將其前處理高密度脂蛋白(25-100 μg/ml)兩小時,接著培養於 0.5mM 的棕櫚酸。研究中使用了 MTT 觀察細胞的存活率,同時也利用了 DCF、DHE 及 Mit.sox 觀察是否有氧化壓力的產生,在蛋白質層面利用了西方墨點法觀察氧化壓力所引起的分子機制。結果顯示,高密度脂蛋白可以減少棕梠酸對心肌細胞造成的凋亡,並且可以抑制棕梠酸對心肌細胞造成的氧化壓力和 JNK/NFκB 訊息途徑,同時也可減少 NFκB 所調控的發炎分子。同時,我們也發現棕櫚酸會減少 BCL_2 家族的蛋白表現,也會造成粒腺體膜電位的不穩定,進而使細胞色素 c 由粒線體釋出於細胞質造成心肌細胞的凋亡現象,而高密度脂蛋白可以有效的減緩這些現象並抑制凋亡的情形。由以上的研究

顯示，高密度脂蛋白可以有效的減少游離的脂肪酸對心肌細胞所造成

功能損傷。

Abstract

High levels circulating saturated fatty acids are associated with diabetes, obesity and hyperlipidemia. In heart, the accumulation of saturated fatty acids has been determined to play a role in the development of heart failure and diabetic cardiomyopathy. High-density lipoprotein (HDL) has been reported to possess key atheroprotective biological properties, including cellular cholesterol efflux capacity, anti-oxidative and anti-inflammatory activities. However, the underlying mechanisms are still largely unknown. Therefore, the aim of the present study is to test whether HDL could protect palmitic acid (PA)-induced cardiomyocyte injury and explore the possible mechanisms. H9c2 cells, a permanent cell line derived from rat cardiac tissue, were pretreated with HDL (50-100 μg/ml) for 2 hours followed by PA (0.5 mM) for indicated time period. Cell viability was determined by MTT assay. The generation of ROS was determined by using the fluorescent probe 2', 7'-dichloro-fluorescein acetoxymethyl ester (DCF-AM), dihydroethidium (DHE), and Mit.sox. The protein levels related to ROS-mediated signaling pathways were measured by Western blot. Our results showed that HDL inhibited PA-induced cell death in a dose-dependent manner. HDL also could rescue PA increased ROS generation and induced the phosphorylation of JNK which in turn activated NF-κB-mediated inflammatory proteins expressions. We also found that PA impaired the balance of BCL_2 family proteins, destabilized mitochondrial membrane potential, and triggered subsequent cytochrome c release into the cytosol and activation of caspase 3. These detrimental effects were ameliorated by HDL treatment.

Results from this study may provide insight into a possible molecular mechanism underlying HDL suppression of the free fatty acid-induced cardiomyocyte apoptosis.

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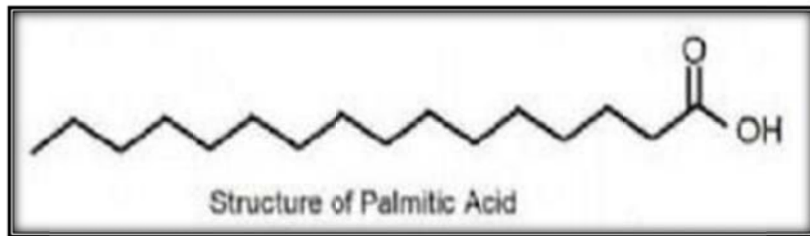
Introduction

1. The progression of diabetic cardiomyopathy

Heart disease and cardiovascular disease are the top ten factors of death in Taiwan (Department of Health, Executive Yuan, R.O.C. (TAIWAN)). Atherosclerosis is considered to be a form of chronic inflammation and a disorder of lipid metabolism (Madamanchi, Vendrov et al. 2005), among the many genetic and environmental risk factors that have been identified by epidemiologic studies elevated levels of serum cholesterol, low levels of HDL, diabetes mellitus, metabolic syndrome, and insulin resistance are probably unique in being sufficient to drive the development of atherosclerosis in human and experimental animals, even in the absence of other known risk factors (Glass and Witztum 2001). High levels of circulating saturated fatty acids are associated with diabetes, obesity and hyperlipidemia. In heart, the accumulation of saturated fatty acids has been proposed to play a role in the development of heart failure and diabetic cardiomyopathy (Zhou, Grayburn et al. 2000; Chiu, Kovacs et al. 2001; Grynberg 2005) as well as ischemia–reperfusion (Grynberg 2005). Palmitic acid, a 16-carbon saturated fatty acid ($CH_3(CH_2)_{14}COOH$), found in animals and plants, which is a major circulating saturated fatty acid. Meat, dairy products, palm oil, and several other plant oils, including soybean, peanut oils contain very small amounts of palmitic acid.

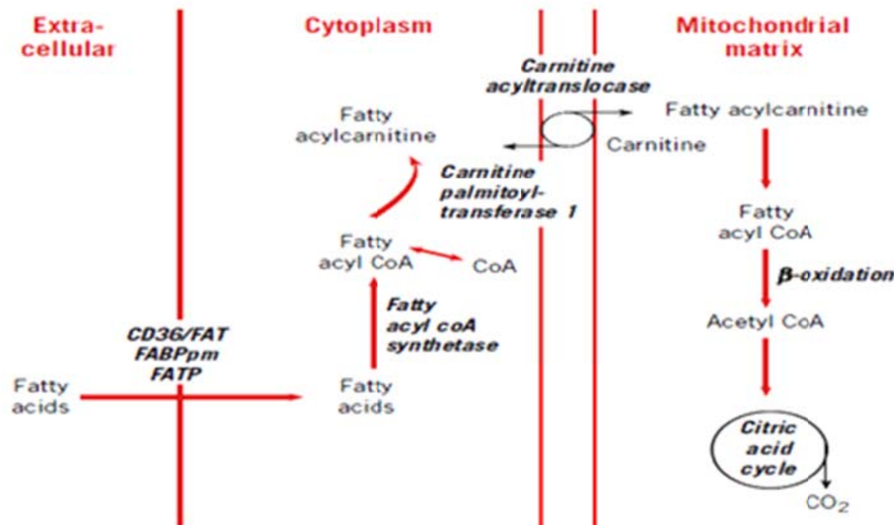


APP.1 The structure of palmitic acid

Excessive palmitic acid has been implicated in the induction of apoptosis in a large variety of cell types including cardiomyocytes (de Vries, Vork et al. 1997; Paumen, Ishida et al. 1997; Listenberger and Schaffer 2002). The World Health Organization claims there is convincing evidence that dietary intake of palmitic acid increases risk of developing cardiovascular diseases (World Health Organization, Geneva, 2003, p. 88). In daily food, Meat, olive oil, corn, peanut oil, cheese, and soybean oil are the sources of palmitic acid.

2. Cardiac fatty acid metabolism pathway

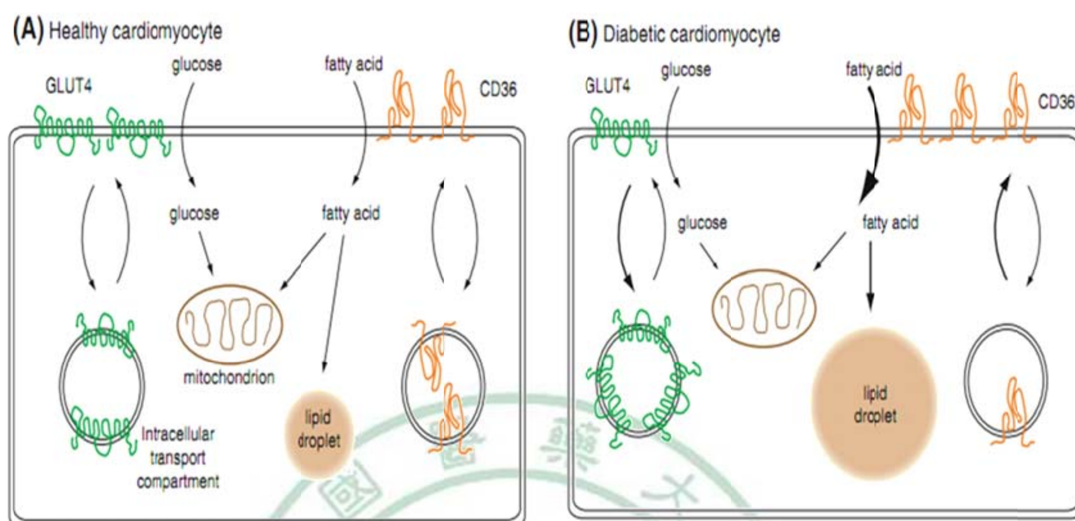
In daily diets, the foods were absorbed via small intestine, and transferred via lipoproteins, lymphatic system. Finally, the foods were digested into glycerol and fatty acids.



APP.2 Overview of fatty acid β-oxidation in the heart.

Fatty acids utilized for cardiac fatty acid β-oxidation primarily originate from either plasma fatty acids bound to albumin or from fatty acids contained within chylomicron or very-low-density lipoproteins (VLDL) triacylglycerol (TAG). Fatty acids are taken up by the heart either via diffusion or via CD36/FATP transporters. Then the intracellular fatty acids are transported into the mitochondria and triggers a diversity of physiological functions. The APP.2 indicates the alterations of transporter localization in diabetic cardiomyocytes compared to healthy cardiomyocytes. In physiological situation, circulating plasma insulin concentrations and increased cardiac work are the most important factors to quickly alter cardiac substrate utilization via reversible translocation of GLUT4 and CD36 from intracellular membrane compartments to the sarcolemma (APP. 2a) (Glatz, Bonen et al. 2006; Glatz, Luiken et al. 2010). Howerer, as shown in APP.2B, substrate uptake in diabetic cardiomyocytes, the localization of GLUT4 and CD36 permanently alters. CD36 presence at the sarcolemma increased fatty acid uptake. In addition,

which may ultimately lead to cardiac metabolic lipotoxicity, and subsequent development of diabetic cardiomyopathy (Steinbusch, Schwenk et al. 2011).



APP.3 Alterations in transporter localisation in diabetic cardiomyocytes compared to healthy cardiomyocytes

3. Palmitic acid-induced cardiomyocyte lipotoxicity and apoptosis

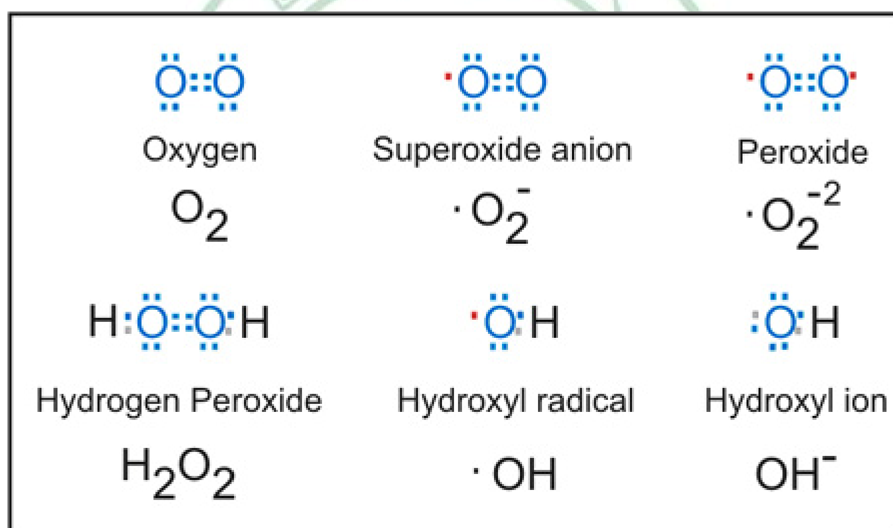
Excess lipid may be stored as triglycerides, but are also shunted into non-oxidative pathways that disrupt normal cellular signaling leading to organ dysfunction and in some cases apoptosis, a process termed lipotoxicity (Wende and Abel 2010). Evidence is elevated levels of serum free fatty acid (FA) levels contribute to the pathogenesis of the metabolic syndrome and heart disease (Listenberger, Han et al. 2003). Acute toxicity from accumulation of long-chain fatty acids may lead to cell dysfunction or cell death (Schaffer 2003). In a variety of experimental systems, all suggest that lipotoxicity from accumulation of long chain fatty acid is specific for saturated fatty acids. In heart, the accumulation of saturated fatty acids has been proposed to play a role in the development of heart failure and diabetic cardiomyopathy as well as

ischemia–reperfusion (Zhou, Grayburn et al. 2000; Chiu, Kovacs et al. 2001). There are multiple pathways can be involved in the acute and chronic cellular effects of NEFA (non-esterified fatty acid) excess, such as reactive oxygen species production, mitochondrial permeability transition pore opening, IκB kinase and NF-κB activation, finally leading to cell dysfunction, apoptosis or necrosis (Weinberg 2006). Apoptosis of cardiomyocytes was shown recently to play a central role in the development of heart failure (Olivetti, Abbi et al. 1997). Saturated fatty acid–induced apoptosis has been demonstrated in neonatal rat cardiomyocytes (de Vries, Vork et al. 1997; Hickson-Bick, Buja et al. 2000; Sparagna, Hickson-Bick et al. 2000).

4. Palmitic acid-induced oxidative stress in cardiomyocytes

Oxidative stress is caused by reactive oxygen species (ROS), also termed oxygen free radicals, are molecules containing unpaired electrons, which is derived from many cellular enzyme systems within the cardiovascular system (Fearon and Faux 2009). ROS are involved in inflammation, endothelial dysfunction, cell proliferation, migration and activation, extracellular matrix deposition, fibrosis, angiogenesis, and cardiovascular remodeling, important processes contributing to cardiovascular and renal remodeling in hypertension, atherosclerosis, diabetes, cardiac failure, and myocardial ischemia-reperfusion injury (Pawlak, Naumnik et al. 2004; San Martin, Du et al. 2007). The major enzymes responsible for ROS generation in the vasculature include NAD(P)H oxidase, xanthine oxidase, mitochondrial autooxidations,

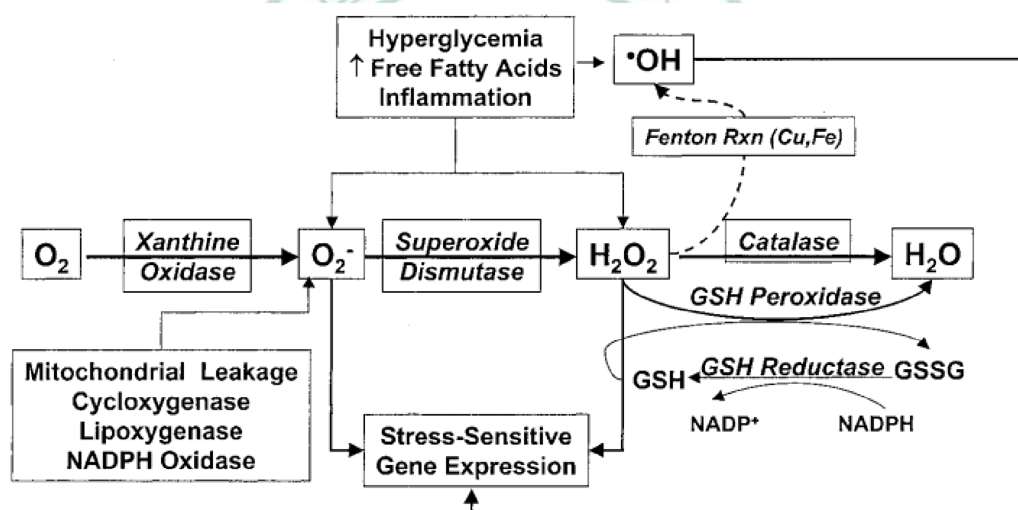
lipoxygenase, and uncoupled NOS. NADPH oxidase was initially identified in phagocytes and it exists as a multisubunit enzyme complex consisting of membrane subunits ($p22^{phox}$ and $gp91^{phox}$) which are the major components responsible for enzyme stability and activity, and at least four cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac1 or 2) which translocate to the membrane when enzyme was activated (Ray and Shah 2005). The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including: superoxide; hydrogen peroxide; hydroxyl radical; hydroxyl ion; and nitric oxide (App.4).



APP.4 Electron structures of common reactive oxygen species. The · designates an unpaired electron.

Antioxidant defense systems may be generally classified into indirect enzymatic antioxidant enzymes and into small molecular weight molecules which directly scavenge free radicals and related reactants. The antioxidant enzymes are regulated by multiple factors, it's represent a first line of defense against these toxic reactants by metabolizing them to

innocuous byproducts. The first enzymatic reaction in the reduction pathway of oxygen occurs during the dismutation of two molecules of $O_2^{\cdot -}$ when they are converted to hydrogen peroxide (H_2O_2) and diatomic oxygen. The enzyme at this step is one of two isoforms of superoxide dismutase (SOD); CuZnSOD is present in the cytosol while MnSOD is located in the mitochondrial matrix. Two enzymes participate in the removal of H_2O_2 from the cellular environment, peroxidases and catalase (Rodriguez, Mayo et al. 2004). APP. 5 showed exogenous and endogenous stimuli leading to ROS generation and activation of stress-sensitive gene expression (Evans, Goldfine et al. 2002).



APP. 5 Exogenous and endogenous stimuli leading to ROS generation and activation of stress-sensitive gene expression.

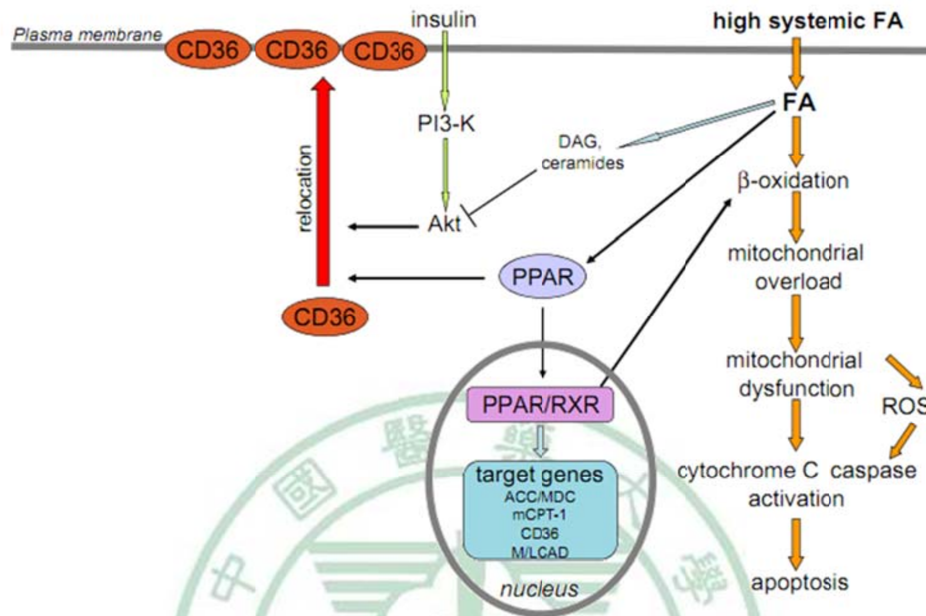
An imbalanced production of ROS plays a role in the pathogenesis of a number of human diseases such as ischemia/reperfusion injury, atherosclerosis, cancer, and allergy. Oxidative stress induced by free fatty acids (FFAs) plays a key role in the development of cardiovascular diseases in metabolic syndrome (Madamanchi and Runge 2007). Elevated

FFA can cause oxidative stress due to increased mitochondrial uncoupling (Wojtczak and Schonfeld 1993; Carlsson, Borg et al. 1999) and β-oxidation (Rao and Reddy 2001; Yamagishi, Edelstein et al. 2001), leading to the increased production of ROS and the activation of stress-sensitive signaling pathways.

5. Sinaling pathway in palmitic acid-induced cardiomyocyte dysfunction

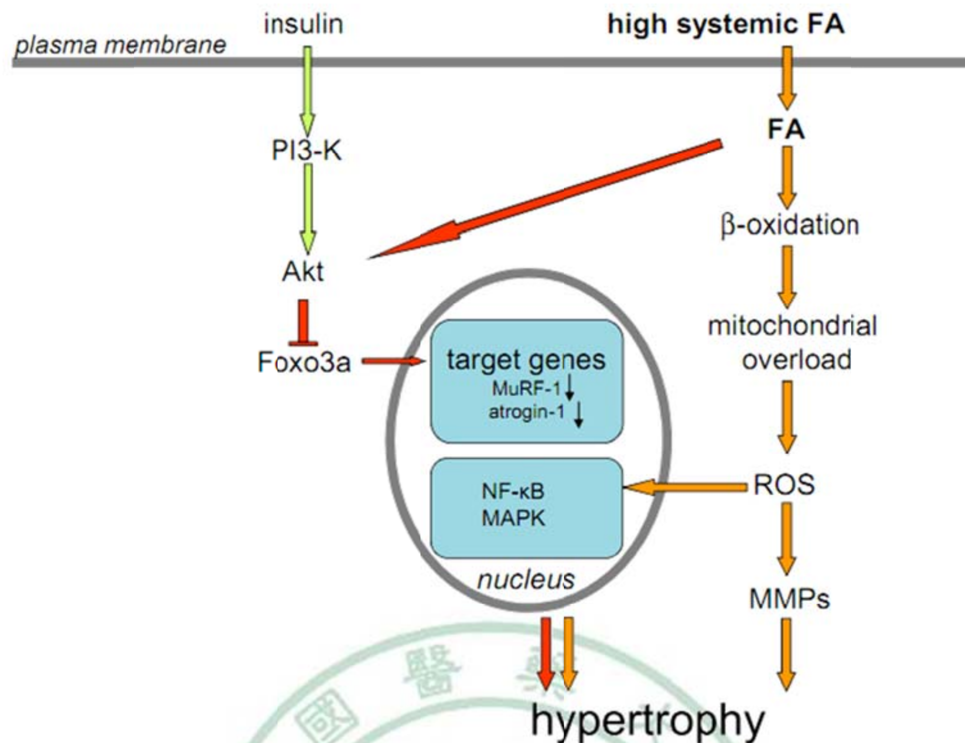
ROS are not only toxic consequences of cellular metabolism but also participants in many intracellular signaling pathways leading to changes in gene transcription and protein synthesis (Griendling, Sorescu et al. 2000). Exposure to a high fat diet induces cardiac contractile dysfunction, which is associated with a permanent relocation of CD36 to the plasma membrane (Ouwens, Diamant et al. 2007). Furthermore, relocation of CD36 appears to be a general phenomenon in insulin resistant hearts (Carley, Atkinson et al. 2007), and precedes cardiac contractile dysfunction (Ouwens, Diamant et al. 2007). In the diabetic heart, mitochondrial oxidative stress induces apoptosis by release of cytochrome c and upregulation of caspase-3 and caspase-9 (Cai, Li et al. 2002; Li, Zhang et al. 2009). In APP.6, high plasma FA concentrations lead to an increase in cytoplasmic FA levels. This stimulates β-oxidation and also stimulates CD36 relocation to the plasma membrane (via PPAR or via increased basal Akt phosphorylation), which leads to an even further increase in cellular FA uptake. The increased intracellular FA concentration causes mitochondrial overload, leading to dysfunction and

oxygen radical production. Eventually, this results in cytochrome c leakage and in caspase activation, which induces apoptosis.



APP.6 Schematic depiction of consequences of increased FA uptake by cardiomyocytes (metabolic remodeling).

Akt signaling is an important regulator of cardiac growth, and its overexpression leads to enhanced contractility, cell survival and pathological cardiac hypertrophy (Condorelli, Drusco et al. 2002; Shioi, McMullen et al. 2002). Such changes are the result of a genetic reprogramming often started by oxidative stress. ROS has been reported as a product of excessive FA oxidation in diabetic cardiomyocytes (APP.6).



APP.7 Schematic depiction of the consequences of increased FA uptake by cardiomyocytes (hypertrophic remodeling).

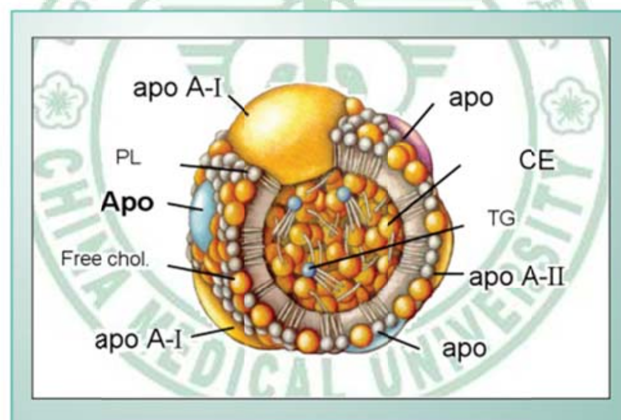
As second messenger, ROS can mediate hypertrophic signals by regulating various intracellular signal transduction cascades and the activity of various transcription factors, such as NF-κB and activator protein-1 (Hirotani, Otsu et al. 2002), and by activating mitogen activated protein kinases(MAPKs) (Nishida, Maruyama et al. 2000; Molkentin 2004) (APP.7). NF-κB, which is a collective term to describe the Rel family of transcription factors, inhibits apoptosis by up-regulating several anti-apoptotic genes (Latella, Sacco et al. 2001). NF-κB is a large number of genes that control cell survival and differentiation including various pro-inflammatory cytokines, chemokines, and adhesion molecules. Many of these same pro-inflammatory molecules, including cytokines such as tumor necrosis factor (TNF) α and interleukin-1(IL-1), are able to activate NF-κB, initiating a signaling cascade of activation. The recent

identification of molecules, which regulate the activation of the NF-κB heterodimer, RelA(p65) and p50 has enhanced our understanding of the molecular mechanisms controlling inflammation. Signaling systems induced by a variety of stimuli activate two serine kinases, termed IκB kinase (IKK)α and IKKβ (or IKK1; IKK2), which target the inhibitors of κB (IκB) (Nakano, Nakajima et al. 2006). Regulation of cell death and survival is also controlled in part by another signaling cascade activated by the mitogen-activated protein kinase(MAPK), which is induced following cellular stress or cytokine signaling (Davis 2000; Kyriakis and Avruch 2001). In mammals, the MAPK cascades are composed of three distinct signaling modules, the c-Jun N-terminal kinase (JNK) cascade, the p38MAPK cascade, and the extracellular signal-regulated kinase (ERK) cascade. Conversely, the c-Jun N-terminal kinase (JNK) promotes apoptosis when activated for prolonged periods (Nakano, Nakajima et al. 2006). Prolonged activation has been shown to be caused by exposure to ROS directly as well as by inactivating JNK inhibitors such as MAP Kinase phosphatases (Nakano, Nakajima et al. 2006). Suppression of TNF-α-induced ROS accumulation seems to be the mechanism by which NF-κB downregulates JNK activation.

6. High density lipoprotein

Lipoproteins consist of lipids and proteins called apoproteins (apo). They are classified according to size, density, and lipid and apoprotein composition: chylomicron (CHY), very low-density lipoprotein (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (Forti and Diament 2006). HDL are

a fraction of serum lipoproteins characterized by similar molecule density (1.063d1.21µg/ml) and size (5–17 nm in diameter) (Nofer, Kehrel et al. 2002). HDL particles consist of 50% apoproteins (AI in larger quantity, AII, CI, CII, CIII, E, and J), 20% of free cholesterol (FC) and esterified (CE) cholesterol, 15% of phospholipids (PL), and 5% of triglycerides (TG) (APP.4) (Forti and Diament 2006). These apolipoproteins play a key role in maintaining the structural integrity of HDL particles but equally exert a major influence on the intravascular metabolism and function of HDL. Of these, apo A-I is most intimately associated with the anti-atherogenic activities of HDL (Chapman 2006).



APP .8 Composition of high-density lipoprotein (HDL).

Furthermore, HDL-C levels < 35 mg/dl are considered low for men, and < 45 mg/dl low for women (1993; Assmann, Cullen et al. 1999). Updated guidelines established that values < 40 mg/dl are undesirable and increase risk for coronary atherosclerotic disease(CAD), whereas values > 60 mg/dl are “protective” (2001; Grundy, Cleeman et al. 2004). Several epidemiologic studies have demonstrated that HDL-C is a strong, independent, inverse predictor of coronary heart disease risk (Gordon,

Castelli et al. 1977; Castelli, Garrison et al. 1986; Assmann, Schulte et al. 1996; Sharrett, Ballantyne et al. 2001). HDL promotes the mobilization and clearance of excess cholesterol via the series of reactions collectively termed "reverse cholesterol transport" (Toth 2009). Another mechanism cited is that HDL possesses such as antioxidant capabilities, anti-inflammatory, anti-thrombotic, and anti-apoptotic activity (Chapman, Assmann et al. 2004).

The effects of dietary fats on the risk of coronary artery disease have traditionally been estimated from their effects on serum total cholesterol (Keys, Anderson et al. 1957; Hegsted, McGandy et al. 1965). A direct atherogenic effect of TG-rich particles, particularly intermediate-density lipoprotein, and remnant particles has been presumed. In a more recent analysis, adjustment for established coronary risk factors, especially HDL cholesterol, substantially attenuated the magnitude of risk associated with high TG levels (Sarwar, Danesh et al. 2007). Therefore, the aim of this study was to explore the mechanisms underlying HDL protects against palmitic acid-induced oxidative stress in cardiomyocytes. We investigated the ROS-mediated NF-κB activation and subsequent inflammatory and apoptotic signaling pathways.

Materials

40%Acrylamide/Bis solution 29:1 (SERVA, Germany)

Annexin V-FITC Apoptosis Detection Kit (BioVion, USA)

Ammonium persulfate/APS (USB, USA)

beta-mercaptoethanol (Pharmacia Biotech, Sweden)

Bovine serum albumin/BSA (Sigma, USA)

Bromophenol Blue (Sigma, USA)

Cosmic Calf Serum/CCS (Hyclone, USA)

DAPI (Sigma, USA)

DCF-AM (2',7'-dichlorofluorescein acetoxymethyl ester; Molecular Probes, Eugene, OR)

Dihydroethidium(DHE)

DMEM (Dulbeccco's Modified Eagle's Medium; Sigma, USA)

DMSO (dimethyl sulfoxide; Sigma, USA)

DTT (1,4-Dithio-D, L-threitol; GERBU, Germany)

Dual-Luciferase®Reporter Assay system (Promega, USA)

ECL kit (enhanced chemiluminescent detection system;Millipore, MA, USA)

Ethylenediaminetetraacetic acid/EDTA (Sigma, USA)

FBS (Fetal bovine serum; GIBCO, USA)

Glucose (USB, USA)

Glycine (Sigma, USA)

Glycerol (Amresco, USA)

Mem-PER®Eukaryotic Membrane Protein Extraction Reagent Kit (PIERCE, 89826)

ME-PER®Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo, 78833)

Methanol 20L (慕容科技有限公司/Taiwan)

Mitochondria Isolation Kit for Cultured cells (Thermo, 89874)

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-terazolium bromide; Sigma, USA)

Neonatal Rat Cardiomyocyte Isolation Kit (Cellutron Life Technology, MD, USA)

Paraformaldehyde (Sigma, USA)

PBS (GIBCO, New Zealand)

Penicillin (Sigma, USA)

Protease inhibitor cocktail tablets (Roche, Germany)

Protein maker (Fermentas)

PVDF membrane pore size 0.45 m (Millipore, USA)

Sodium dodecyl sulfate /SDS (Sigma, USA)

Sodium chloride/NaCl (Sigma, USA)

Sodium bicarbonate/$NaHCO_3$ (Sigma, USA)

TEMED (Sigma, USA)

Tris(USB, USA)

Tris-base (USB, USA)

Tris-HCl (USB, USA)

Triton X-100(TEDIA, USA)

TUNEL (Terminal deoxynucleotide dUTP-biotin nick-end labeling; Roche, Mannheim, Germany)

Tween 20(Pharmacia, Sweden)

Trypsin-EDTA (GIBCO, USA)

脫脂奶粉(安佳, New Zealand)

Antibody	
1st Ab	Brand
p-Akt (Ser473)	Cell Signaling
β-actin (C4)	Santa Cruz
Bax (P-19)	Santa Cruz
BCL_2	BD
Caspase-3 (H-277)	Santa Cruz
Cox-2	Cell signaling
Cox IV (Va)	Invitrogen
p-ERK 1/2 (E-4)	Santa Cruz
Flotinllin	Cell Signaling
IKKα/β (H-470)	Santa Cruz
IκBα (H-4)	Santa Cruz
p-IKKα/β (Ser176)	Santa Cruz
p-IκB-α (Ser32) (14D4)	Cell signaling
p-SAPK/JNK (Thr183/Tyr185)	Cell Signaling
MMP-3	
NFκB p65 (A)	Santa Cruz
Nitrotyrosine (HM11)	Biomol
NOX-2/gp91 phox	abcam
p-NF-κB p65 (Ser536) (93H1)	Cell Signaling
p22-phox (FL-195)	Santa Cruz

p47-phox	upstate
PCNA (FL-261)	Santa Cruz
p-p38 (D-8)	Santa Cruz
Rac1	abcam
SOD-1 (C-17)	Santa Cruz
SOD-2 (MnS-1)	Santa Cruz
anti-mouse IgGhorseradish peroxidase conjugated	Santa Cruz
anti-rabbit IgGhorseradish peroxidase conjugated	Santa Cruz
anti-gout IgGhorseradish peroxidase conjugated	Santa Cruz

Methods

1. Cell culture

H9c2 cell lines were obtained from American Type Culture Collection (ATCC), cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% Cosmic CalfR serum (CCS), 2mM glutamine, 100units/ml penicillin, 100μg/ml streptomycin, and 1mM pyruvate in humidified air (5% CO_2) at 37 ^{o}C. During the treatment, pretreated with HDL for 2 hours and then stimulated with palmitic acid (PA) for 24 hours. The specificity of the inhibit ROS and mitochondria complex Ⅰ inhibitor by adding N-acetly cysteine (NAC) (500µM).

2. Lipoprotein separation

Human plasma was obtained from the Taichung Blood Bank (Taichung, Taiwan) and HDL was isolated using sequential ultracentrifugation (=1.019-1.063 g/ml) in KBr solution containing 30 mM EDTA, stored at 4℃ in sterile, dark environment and used within 4 days as previously described. HDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 Mgel (Pharmacia) in 0.01 mol/l phosphate-buffered saline (136.9 mmol/l NaCl, 2.68 mmol/l KCl, 4 mmol/l Na_2HPO_4, 1.76 mmol/l KH_2PO_4) at pH 7.4. Protein concentration was determined by Bradford Protein Assay.

3. MTT assay

MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide]. The H9c2 cells were inoculated into 24-well plate. After HDL and palmatic acid treatments, the medium was removed and MTT solution (0.5mg/ml) was added to each well which containing cells, subsequently incubated the plate in a 5% CO_2 incubator at 37ºC for 1 hour. MTT solution was replaced by isopropanol to dissolve blue formazan crystals, and absorbance was measured at 570 nm by using a microplate reader.

4. DAPI staining and TUNEL assay

After various treatments, H9c2 cells grown on 6 mm plate were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with PBS, cells were treated with permeation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4ºC. Following wash with PBS, samples were first incubated with Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL) reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate-dUTP. The cells were also stained with 1μg/ml DAPI for 30 min to detect cell nucleus by UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope, respectively. Apoptotic cells were assessed by fluorescence microscope or in a flowcytometer.

5. Reactive oxygen species and mitochondrial superoxide production

Intracellular ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes), dihydroethidium (DHE) and MitoSOX™ as a probe for the presence of H_2O_2 or superoxide. DCFH-DA is converted by intracellular esterases to DCFH, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant, and then analyzed by flow cytometry. Dihydroethidium (DHE), by virtue of its ability to freely permeate cell membranes is used extensively to monitor superoxide production. It had long been postulated that DHE upon reaction with superoxide anions forms a red fluorescent product (ethidium) which intercalates with DNA. DHE is perhaps the most specific and least problematic dye; as it detects essentially superoxide radicals, is retained well by cells, and may even tolerate mild fixation. MitoSOX™ Red mitochondrial superoxide indicator is a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells, which is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence. MitoSOX™ is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species (RNS)–generating systems, and oxidation of the probe is prevented by superoxide dismutase. The oxidation product becomes highly fluorescent upon binding to nucleic acids.

6. Immunoblotting

Culture H9c2 cells were scraped and washed once with PBS, then cell suspension was spun doen, and lysed in RIPA buffer (HEPES 20mM, MgCl2 1.5mM, EDTA 2mM, EGTA 5mM, dithiothreitol 0.1mM, phenylmethylsulfonyl fluoride 0.1mM, pH 7.5), and spun down 12,000 rpm for 20 min, the supernatant was collected in new eppendorf tube. Proteins (30 μg) were separated by electrophoresis on SDS-polyacrylamide gel. After the protein had been transferred to polyvinylidene difluoride membrane, the blots was incubated with blocking buffer (1X PBS and 5% nonfat dry milk) for 1 hour at room temperature and then probed with primary antibodies (1:1000 dilutions) overnight at 4°C , followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody at a 1:50000 dilution. The bound immunoproteins were detected by an ECL kit.

7. Measurement of mitochondria membrane potential

The lipophilic cationic probe fluorochrome 5,5',6,6'-tetrachloro1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was used to explore the effect HDL on the mitochondria membrane potential (ΔΨm). JC-1 exists either as a green fluorescent monomer at depolarized membrane potential or as a red fluorescent J-aggregate at hyperpolarized membrane potential. JC-1 exhibits potential-dependent accumulation in mitochondria, as indicated by the fluorescence emission shift from 530 to 590 nm. After treating cell

with palmitic acid (0.5mM) for 24 hours in the presence or absence various concentrations of HDL, cell ($5X10^4$ cell/24-well plates) were rinsed with DMEM, and JC-1 (5μM) was loaded. After 20 min of incubation at 37 ℃ , cell were examined under a fluorescent microscope. Determination of the $\Delta\Psi m$ was carried out using a FACScan flow cytometer.

8. Isolation of cytosolic fraction for cytochrome c analysis

After treating cells with palmatic acud in the presence and absence of natural products, the cells were collected and lysed with lysis buffer (20mmol/L HEPES/ NaOH, pH 7.5, 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 2mmol/L EDTA, 5 mmol/L EGTA, 1 mmol/L DTT, protease inhibitor cocktail) for 20 min on ice. The samples were homogenized 30 strockes by glass Dounce and pestle. The homogenates were then centrifuged at 500x g to remove unbroken cells and nuclei. Supernatant were centrifuged at 17000x g for 30 min to isolate mitochondria fraction. Supernatant was cytoslic extraction and pellet was mitochondria fraction lysed by RIPA buffer. Cytosol and mitochondria protein were resolved by SDS-polyacryamide gel electrophoresis.

9. Nuclear protein extraction

Cells grown to 80% confluency and subjected to various treatments were subsequently washed with ice-cold PBS and it was prepared for nuclear protein extraction. Cells grown on 10-cm dish were gently

scraped with 3 ml ice-cold PBS and it were centrifuged at 1,000x g for 10 min at 4°C. After carefully aspirating the supernatant, cells were resuspended with 200μl ice-cold BUFFER-I (10 mM Hepes (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, and proteinase inhibitor cocktail and incubated for 15 min on ice to allow cells to swell, followed by adding 20μl IGEPAL-CA630. After vigorously vortexing for 10 s and centrifuging at 16,000 g for 5 min at 4°C, the supernatant (cytoplasmic fraction) were carefully aspirated and the pellet were resuspended with ice-cold BUFFER-II (20 mM Hepes (pH 8.0), 1.5 mM $MgCl_2$, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol and proteinase inhibitor cocktail and vigorously vortex. After vortexing, the suspension was placed on ice for 30 min before centrifuging at 16,000x g for 15 min at 4°C. The supernatants (nuclear extracts) were stored aliquots at -80°C. Protein concentration of the supernatants was determined by the colorimetric assay.

10. Transfection luciferase or siRNA assay

Transient transfections were carried out by the proprietary cationic polymer reagent (Fermentas) (TurboFect™ *in vitro* Transfection Reagent) following the manufacturer's instruction. In some experiments 2×10^4 cells were plated onto 24-well plates and grown overnight. Vectors, including the reporter vectors, and the internal Renilla luciferase control vector (0.1 μg), and other protein expression vectors were cotransfected as indicated in the figure legends. All assays for firefly and Renilla luciferase activity were performed using one reaction plate sequentially. Briefly, at 24 h post-transfection and

stimulation, the cells were washed with phosphatebuffered saline and lysed with Passive Lysis Buffer. After a freeze/thaw cycle, samples were mixed with Luciferase Assay Reagent II, and the firefly luminescence was measured with a Luminometer. Next, samples were mixed with the Stop & Glo reagent, and the Renilla luciferase activity was measured as an internal control and to normalize the luciferase activity values. Double-stranded siRNA sequences targeting JNK, NF-κB mRNAs were obtained from Santa Cruz Biotechnology. The non-specific siRNA (scramble) consisted of a nontargeting. Cells were cultured in 60-mm well plates in medium. Transfection of siRNA was carried out with transfection reagent. Specific silencing was confirmed by immunoblotting with cellular extracts after transfection.

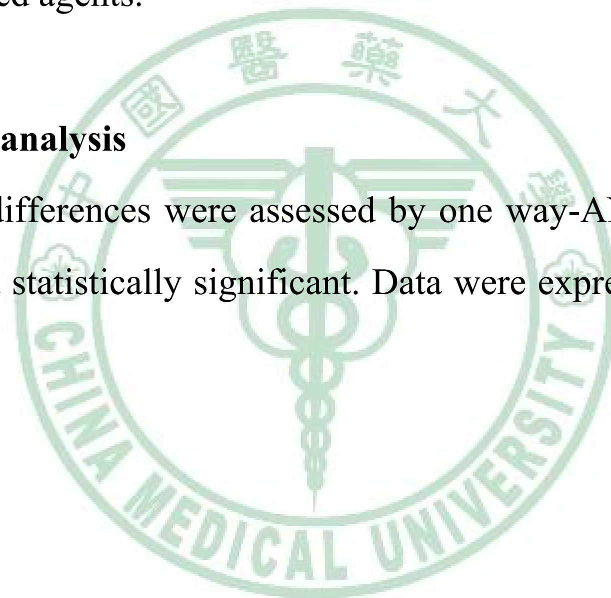
11. Annexin V-FITC/PI Staining

H9c2 cells seeded at a density of 2×10^5 cells/well in 6-well plates were exposed to hypoxia for 24 h. Apoptotic cells were monitored by FACSCanto flow cytometry using the Annexin V-FITC Apoptosis Detection Kit. Total cells and supernatants were collected, washed and incubated for 15 min with 1 × binding buffer containing annexin V-conjugated fluorescein isothiocyanate (FITC) and propidium iodide (PI). Annexin V positive cells were considered as early apoptotic cells. Cells with annexin V and PI positive were considered as late apoptotic and/or necrotic cells whereas viable cells were unstained.

12. Cardiomyocyte Culture

Neonatal cardiomyocytes were isolated and cultured using the

commercial Neonatal Cardiomyocyte Isolation System Kit according to manufacturer’s directions. Briefly, hearts from one- to two-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion solution at 37 °C. Ventricular cardiomyocytes were isolated and cultured in DMEM containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. After 3-4 days, cells were incubated in serum-free essential medium overnight before treatment with indicated agents.



13. Statistical analysis

Statistical differences were assessed by one way-ANOVA. $P < 0.05$ was considered statistically significant. Data were expressed as the mean ± SEM.

Results

Palmitic acid (PA)-induced apoptosis, and cells death.

To clarify palmitic acid induced cytotoxicity in cardiacmyocyte, H9c2 were treated with different concentrations of PA for 24 and 48 h. The result of MTT showed that after treatment with various concentrations of PA for indicated time peroid significantly decreased the cell viability in a dose-dependent manner (Fig.1A). The cell viability is lower then 50% in concentration of PA on 0.5mM treated with H9c2 cells ,therefore 0.5 mM was used for the following experiments.We also used TUNEL analysis for observing cells undergoing apoptosis. After incubation with PA for 24 h, we observed a significant increase apoptosis bodies (Fig.1B).

Palmitic acid increased generation of mitochondrial reactive oxygen species (ROS).

Previous investigation demonstrated that free fatty acid (FFA) induced-oxidative stress plays an important key role in development of cardiovascular disease in metabolic syndrome (Madamanchi and Runge 2007). We therefore, examined the cellular ROS levels after treatment with 0.5 mM PA for 24 h by fluorometric assay using DCF-AM and DHE. As shown in Fig 2A and 2B, an approximately three-fold and two-fold increase of ROS and superoxide was observed in cells incubated with PA compared with untreated cells. NADPH oxidase and mitochondrion are known major sources of superoxide (Land 2012), so we measured the expression levels of NADPH oxidase subunits by using Western blot (Fig.2D) and generation of superoxide in mitochondira by using

MitoSOX™ Red (Fig.2C). In Fig 2C, an approximately three-flod increase of mitochondrial superoxide was observed in cells incubated with 0.5 mM PA for 24 h compared with normal condition. However, the protein levels of $gp91^{phox}$, $p47^{phox}$, Rac-1 protein in H9c2 cells in a time dependent manner (0-24h) (Fig.2D).

Intracellular ROS levels are regulated by the balance between ROS generation and antioxidant enzymes such as catalase or SOD. Besides, the involved ROS are able to inactivate antioxidative enzymes that additionally increase the imbalance in favor of oxidative stress. Therefore, we investigated the expression of its isoforms in H9c2 cells in response to PA. Our results showed that the antioxidant enzymes SOD1 and SOD2 decreased in H9c2 cells treatment with PA for 0.5mM (Fig.2E).

Palmitic acid led to collapse of mitochondria member potential

To examine whether influence of mitochondrial disruption accounts for the apoptosis effect of PA, we tested the effect of PA on mitochondrial permeability. When H9c2 cells were exposed to PA (0.5 mM), the $\Delta\Psi m$ was depolarized, quantitative analysis from flow cytometry supported these findings (Fig.3A).

Palmitic acid induced-apoptosis involved in a mitochondrial-dependent pathway.

BCL_2 family proteins are upstream regulators of mitochondrial membrane potential. Since PA depolarized $\Delta\Psi m$, whether PA also influenced Bcl_2 family protein was investigated. After treated PA for different times (0-24 h), the immunobolotting studies demonstrated that

PA downregulated the antiapoptoic (BCL_2 and p-AKT^{ser473}) and upregulated the proapoptoic (Bax) proteins, also increased caspase 3 activity in H9c2 cells (Fig.3B).

It is known that disruption of mitochondrial membrane function results in the discharge of the mitochondrial enzyme cytochrome c into the cytosol. Consequently, mitochondria were separated from the cytosolic fraction and detected by Western blotting. As show in Fig.3C, the amount of cytochrome c released into the cytosolic fraction was much greater in H9c2 cells that had been incubated with PA for 24 h than in control cells. The results show that PA significantly induced release of cytochrome c.

Role of MAPK family proteins, NFκB signaling pathway in PA induced apoptosis.

To investigate whether MAPK family protiens were involved in the apoptosis-related signaling pathways activated in H9c2 treated with PA, we examined the expression levels of MAPK family proteins by Western blot. Our results showed that the phosphorylation of JNK, but not ERK or P38 was increased after treatment with PA for 24 h (Fig.4A).

Accumulated evidne indcaed that IκB kinase/NF-κB (IKK/NFκB) signaling pathways play critical roles in a variety of physiological and pathological processes many stimuli activate NF-κB, mostly through IκB kinase–dependent (IKK-dependent) phosphorylation and subsequent degradation of IκB proteins. The liberated NFκB dimers enter the nucleus, where they regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and pro- and antiapoptotic proteins. In accordance with previous findings, our results showed protein

level of NFκB was increasing in the nuclear fraction (Fig.4B). In addition, cells were transfected with construct containing the NFκB-responsive luciferase reporter gene (NFκB Luc) to further confirm the effects of PA on NF-κB activation. The result of lucisferase assay indicated that NFκB promoter activity was increasing in a time-dependent manner (Fig.4C). In order to identify whether JNK is an upstream regulator of NF-κB, we knockdown JNK and NF-κB by using si-RNA (10nM) to advance study PA-induced apoptosis pathway. Cells were transfected with si-RNAs for 24 h and then treated with PA plus anti-oxidant (NAC) (500μM) for 24 h, apoptosis could be markedly suppressed by JNK, NFκB, and NAC. The data showed NFκB si-RNA had no effect on PA-induced JNK activity (Fig.4D). These observations indicate that JNK/NFκB pathway could mediate cardiomyocyte cell apoptosis induced by palmatic acid, but NFκB has no influence on JNK activity.

HDL downregulated palmitic acid-induce apoptosis

HDL is a complex, bioactive particle, containing multiple acute phase response proteins, protease inhibitors, and complement regulatory proteins. So, I would like to know whether HDL can downregulated palmitic acid-induce apoptosis in H9c2 cardiomyocytes cells.

The viability of cells incubated with PA in the absence or presence of indicated concentrations of HDL was assessed using the MTT assay (Fig.5A). The result showed that PA significantly reduced viability in H9c2 cells after 24h of incubation; however, pretreatment with HDL inhibited PA-induced cytotoxicity of H9c2 cells dose dependently. Next, we examined whether HDL possesses antiapoptoic effects in PA-treated

H9c2 cells. To further determine whether HDL could protect against PA-induced apoptosis, PA-treated cells were analyzed biochemically via Annexin V binding assay (Fig.5B) and TUNEL and DAPI staining assay (Fig.5C) and evaluated by flow cytometry (Fig.5D) and microscopic observation (Fig.5C). Our results showed that the cells showed typical features of apoptosis, including the formation of compressed nuclei after treated with PA for 24h, which were, however, not observed in the HDL-pretreated H9c2 cells and also reduced the phenomenon of apoptosis in dose dependently. Phase-contrast microscopy was performed to examine the protective effects of HDL on morphological features of H9c2 cells after exposure to PA, the number of shrunken cells of cells with blebbing membranes was significantly reduced by the presence of HDL (Fig.5C) and anti-oxidant (NAC,500μM) (Fig.5D).

HDL inhibits palmitic acid-induce ROS and superoxide generation.

Studies demonstrated that PA induce elevate the concentration of cellular ROS, which subsequently led to the change the cell signaling pathway to mediate cell dysfunction. Therefore, we investigated the effects of HDL on generation of ROS, a potential factor related to PA-induced H9c2 cells injury, by using hydroxyl radical sensitive probe 2',7'-dichlorofluorescein acetoxymethyl ester (DCF-AM)(Fig.6A) and MitoSOX™ Red mitochondrial superoxide indicator (Fig.6B). The levels of ROS and mitochondrial superoxide generation have significantly decreased in H9c2 cells after pretreatment with HDL (25-100μg/ml) for 2h before exposure to PA (0.5 mM) in a dose-dependent manner (Fig.6C), the result were measured by flow cytometry. Intracellular ROS levels are

regulated by the balance between ROS generation and antioxidant enzyme. Besides, the involved ROS are able to inactivate antioxidative enzymes that additionally increase the imbalance in favor of oxidative stress. Therefore, we clarify the expression of SOD isoforms in H9c2 cells in response to PA. The results showed that HDL significantly reduced the suppression of SOD activity caused by PA in dose dependent manner, expression was diminished after treatment with PA for 24h and could be significantly rescued by pretreatment with HDL (25-100μg/ml) (Fig.6D).

Sustain exposures HDL can reduce mitochondrial ROS in neonatal cardiomyocytes treated with PA.

To verify lipotoxicity induced cardic induced cellular ROS generate, and wether HDL could attenuate the phenomenon. We examined the cellular of mitochondrial superoxide generation in cultured primary rat neonatal cardiomyocytes. Pretreatment of neonatal cardiomyocytes with HDL (25-100μg/ml) for 2h before exposure to PA for 24h. We used MitoSOX™ Red mitochondrial superoxide indicator to confirm by microscopic observation (Fig.7A) and flow cytometry (Fig.7A). As show in Fig.7, PA enhance mitochondrial superoxide generation returned to levels close to those seen in control cells when neonatal cardiomyocytes were treated with HDL (25-100μg/ml) to sitmulation with PA, there were also reversed by anti-oxidant (NAC,500μM) and mitochondrial superoxide inhibitor (Rotenone,5μM).

HDL stabilized on mitochondrial transmembrane permeability transition.

To examine whether inhibition of mitochondrial disruption accounts for the anti-apoptotic effect of HDL, we examined the effects of HDL on mitochondrial permeability. When H9c2 cells were exposed to PA (0.5mM), the $\Delta\Psi m$ was depolarized, as shown by the increase in green fluorescence. Pretreatment with HDL reduced the change in $\Delta\Psi m$, as indicated by repression of green fluorescence and restoration of red fluorescence. As shown in Fig.8, PA caused a marked increase in JC-1 green fluorescence (middle) compared with the control (left). Preatreatment with HDL (100μg/ml) caused marked inhibition of this PA-induced apoptotic index (right).

HDL restores surivial protein expression and suppresses caspase 3 activity.

Since PA depolarized $\Delta\Psi m$ whereas HDL maintained it, whether HDL also influenced the equilibrium of Bcl-2 family proteins was investigated. Immunoblotting studies demonstrated that PA downregulated the antiapoptotic and survival protein (Bcl_2, p-AKT^{ser473}), also upregulated the proapoptoic protein (Bax), whereas HDL pretreatment effectively repressed these PA-induced proapoptoic events (Fig.9). Therefore, activated caspase 3 is a key factor in the execution of mithchondrial apoptosis (Narula, Pandey et al. 1999). Wether PA and HDL ultimately influence this factor to modulate apoptosis, we subsequently determined the pro-form and active-form of caspase 3 by immunoblotting (Fig.9).

The data showed that active caspase 3 was significantly increased in cells that had been treated with PA was suppressed in cells that had been pretreated with HDL.

HDL decreased p-JNK and p-NFκB protein expression, and down-regulation of promoter activity in H9c2 cardiomyoblast cells.

It has been shown that the transcriptional factor NFκB can be induced by a multitude of atimuli, including cytokines and ROS. However, in cardiomyocytes, NFκB activation has been found to produce cell apoptosis instead of preventing the cells from apoptosis. Therefore, we sought to determine whether HDL inhibits NFκB-triggered downstream inflammatory proteins in H9c2 cells. We investigated the immunostaining of p-NFκB, p-JNK and NFκB-triggered downstream inflammatory proteins, to determine whether HDL inhibits the phenomenon. As show in Fig.10A, pretreated with HDL (25-100μg/ml) significantly inhibited p-NFκB, p-JNK, COX_2, and MMP-3 protein expression in a dose-dependent manner. Further, the activation of NFκB was measured in terms of its ability to promote of target gene expression. The result of the luciferase assay were used to represent the activity of NFκB regulate the expression of its target genes. Cells transiently transfected with NFκB lucisferase plasmid after 24h were exposed to PA with different concentrations of HDL (25-100μg/ml) for 24h. As shown in (Fig.10B), there was an approximately threefold increase in lucisferase activity in H9c2 cells stimulated with PA as compared with control. Pretreatment with HDL (25-100μg/ml) inhibited PA-induced luciferase activity in a dose-dependent manner. These finding indicate that PA causes activation

of NFκB, and HDL could significantly inhibit NFκB activity.

Discussion

In the present study we found that palmitic acid induced significant apoptosis and ROS generation in H9c2 at the concentrations above 0.5mM. Although some papers were obtained palmitic acid with this ratio, it is different from the most often used 2/1 ratio (de Vries, Vork et al. 1997; Hickson-Bick, Buja et al. 2000; Ostrander, Sparagna et al. 2001).

Dietary fats modify the composition of cellular and mitochondrial membranes (Innis and Clandinin 1981; Lemieux, Blier et al. 2008), affecting their susceptibility to peroxidation by ROS (Pamplona, Portero-Otin et al. 2000). Oxidative stress is recognized as an important trigger in the development of cardiovascular disease (Fearon and Faux 2009). Mitochondrial β-oxidation of fatty acids is the major source of energy for the heart. Mitochondria are also central to stress-induced programmed cell death. In addition, in nonphagocytic cells, these organelles are the principal site of ROS production, via the electron transport chain (Hickson-Bick, Sparagna et al. 2002). Our results showed that plamitic acid-induced ROS generation through mitochondria not from NADPH complex (Fig.2C.D). Antioxidant defense enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase.

There are three types of SOD expressed in blood vessel: cytosolic Cu/Zn-SOD, Mn-SOD localized in mitochondria, and an extracellular form of Cu/Zn-SOD (Faraci and Didion 2004). SOD protects against superoxide-mediated cytotoxicity by rapidly dismutating O^{2-} to H_2O_2. Cu/Zn-SOD, after treated with palmitic acid, decreased Cu/Zn- SOD and Mn-SOD protein expression level (Fig.2E). Palmate-induced apoptosis in

the neonatal cardiomyocyte is associated with a decrease in the mitochondrial membrane potential, also decreasing the ability of the mitochondria to produce ROS (Hickson-Bick, Sparagna et al. 2002). ROS have been implicated in signal transduction pathways leading to a modulation of the DNA-binding activities of the transcription factor NFκB (Maulik, Sasaki et al. 2000), implying a role for alterations in gene transcription as a response to oxidative stress, p38 MAPK, NH2-terminal Jun kinases/stress-activated protein kinases (JNK/SAPK), advanced glycosylation end-products (AGE)/receptor for AGE (RAGE), and protein kinase C (PKC) (Evans, Goldfine et al. 2002).Our results showed that palmitic acid induced ROS generation and decreased mitochondria membrane potential, also downregulated the antiapoptoic (BCL_2 and p-AKT^{ser473}) and upregulated the proapoptoic (Bax) proteins, furthermore, increased caspase 3 activity in H9c2 cells (Fig.3B). Palmitic acid disruption of mitochondrial membrane function results in the discharge of the mitochondrial enzyme cytochrome c into the cytosol. In our study, we found that palmitic acid increased JNK/SAPK protein expression, but not p38 MAPK and ERK (Fig.4A).We assumed that palmitic acid induced-damage through JNK/SAPK dependent pathway. The stress-activated protein kinases JNK1 and IKK are central signal transducers in innate immunity and stress responses that control the expression of several proinflammatory genes (Solinas and Karin 2010). Recently it has become evident that interference with either JNK1 or IKK activity improves insulin signaling in mouse models of obesity and lipid-induced glucose intolerance (Yuan, Konstantopoulos et al. 2001; Hirosumi, Tuncman et al. 2002). Moreover, JNK and IKK are also

downstream of pathways activated by toxic lipids and excessive glucose levels (Solinas and Karin 2010). In addition to JNK activation (Kamata, Honda et al. 2005), oxidative stress was proposed to activate NFκB (Schreck, Rieber et al. 1991; Sen and Packer 1996; Manna, Zhang et al. 1998). However, the link between NFκB and ROS has become complex because NFκB activation has anti-oxidant functions (Li and Karin 1999; Pham, Bubici et al. 2004; Kamata, Honda et al. 2005). JNK activation may, however, promote ROS accumulation (Ventura, Cogswell et al. 2004) and link ROS production to insulin resistance and loss of β-cell function (Kaneto, Matsuoka et al. 2007; Temkin and Karin 2007; Matsuzawa and Ichijo 2008). We therefore used siRNA to figure out whether JNK/ NFκB pathway could mediate cardiomyocyte cell apoptosis induced by palmitic acid. As shown in Fig.4D NFκB has no influence on JNK activation.

Epidemiological and clinical studies have demonstrated the inverse association between HDL cholesterol levels (HDL-C) and the risk of coronary heart disease (CHD) (Gordon and Rifkind 1989; Assmann, Schulte et al. 1996). Low HDL-C is the most frequent dyslipoproteinemia in patients with premature infarction (Genest, Martin-Munley et al. 1992) and is an independent predictor of recurrent coronary events(Bolibar, von Eckardstein et al. 2000; Ridker 2001). Furthermore raising HDL, decreases the incidence of coronary artery disease (Robins 2001). Several different actions are attributed to HDLs, which taken all together, have an anti-atherogenic effect. The primary action is the reverse cholesterol transport, mentioned above. Other actions have been described *in vitro* and in animals, such as: antioxidant, anti-inflammatory, platelet antiaggregant, anticoagulant, profibrinolytic, and endothelial protection

effects (von Eckardstein and Assmann 2000; Nofer, Kehrel et al. 2002; Alsheikh-Ali, Kuvin et al. 2005). In summary, the present results indicated that HDL attenuates palmitic acid-induced cardiomyocyte lipotoxicity and oxidative dysfunction via modulating mitochondria dependent pathway and p-JNK/NFκB signaling (Fig.3, 4). Therefore, reduce the downstream of superoxide-induced ROS generation and impairment of antioxidant enzymes, and inflammatory protein expression (Fig.10). In addition, HDL inhibited palmitic acid-induced cell death and apoptosis in cardiomyocytes (Fig.9). Further studies are required to confirm the effect of HDL on the inhibition of palmitic acid mediated pro-atherogenic effects and the effectiveness in vivo. Our findings may be a relevant therapeutic molecular mechanism in the improvement of cardiovascular disease. In H9c2 cells, several lines of evidence demonstrated that palmitic acid are taken up by the heart either via CD36/FATP transporters (Lopaschuk, Ussher et al. 2010). Whether HDL protects the cells against palmitic acid-induced apoptosis via Apo A-1, SR-B1 or other receptors will be another issue we can identify in the future study.

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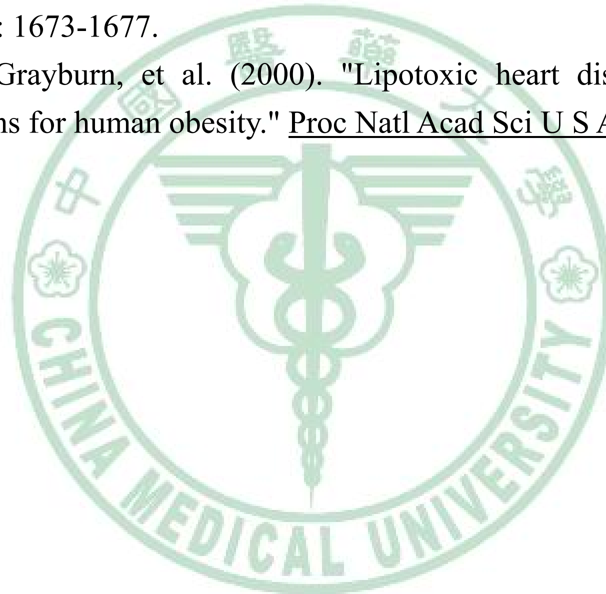


Figures

Fig.1

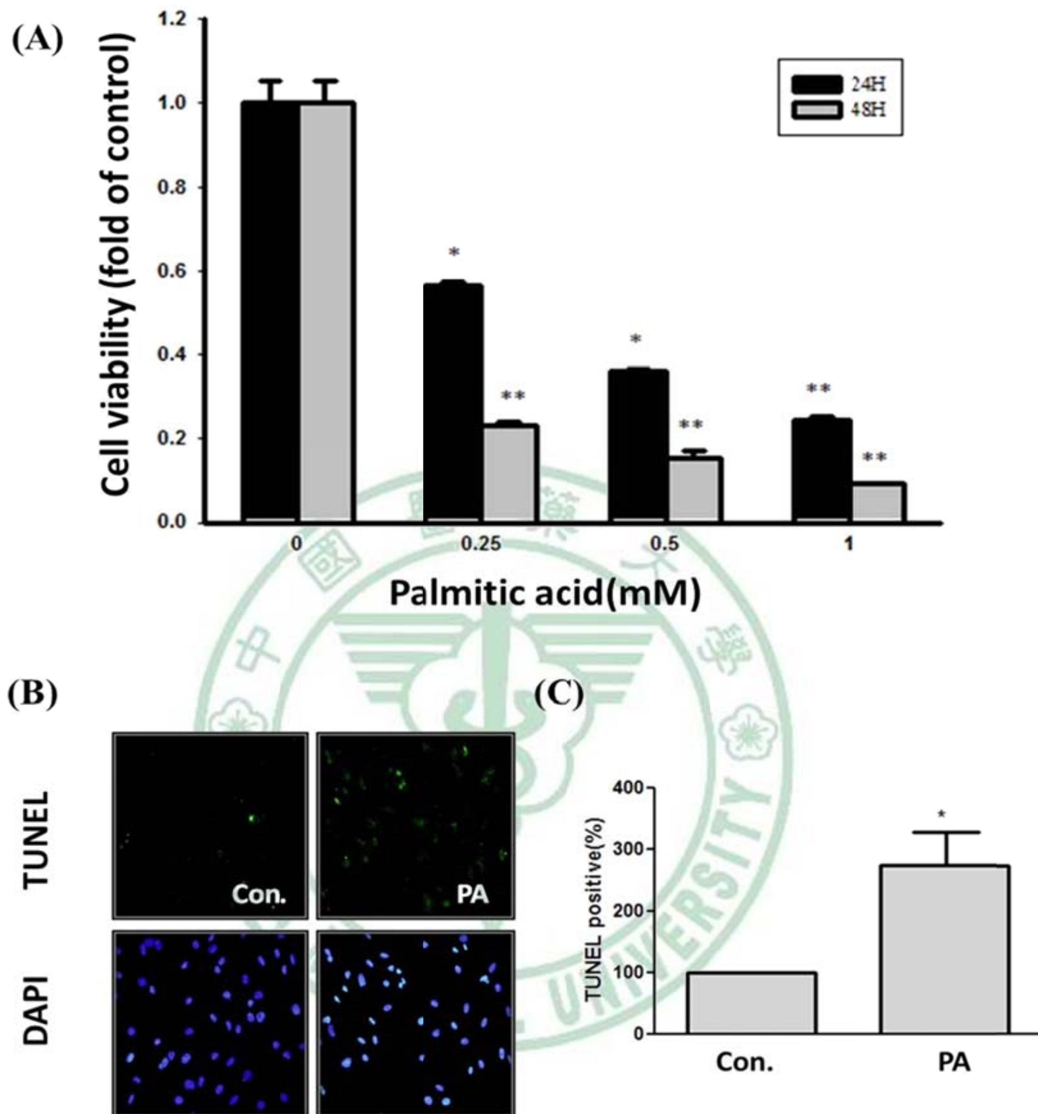


Fig.1 Effects of palmitic acid (PA)-induced H9c2 cells death. (A) Cells viability was measured by MTT assay. H9c2 cells were incubated with PA at different concentrations for 24 or 48 h. (B and C) Fluorescence images show the cells stained with 4,6-diamidino-2-phenylindole (DAPI) and stained using terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay. H9C2 cells were incubated with PA (0.5mM) for 24 h. Data showed the means±SEM of 3 independent analyses.*P<0.05 compared with control.

Fig.2

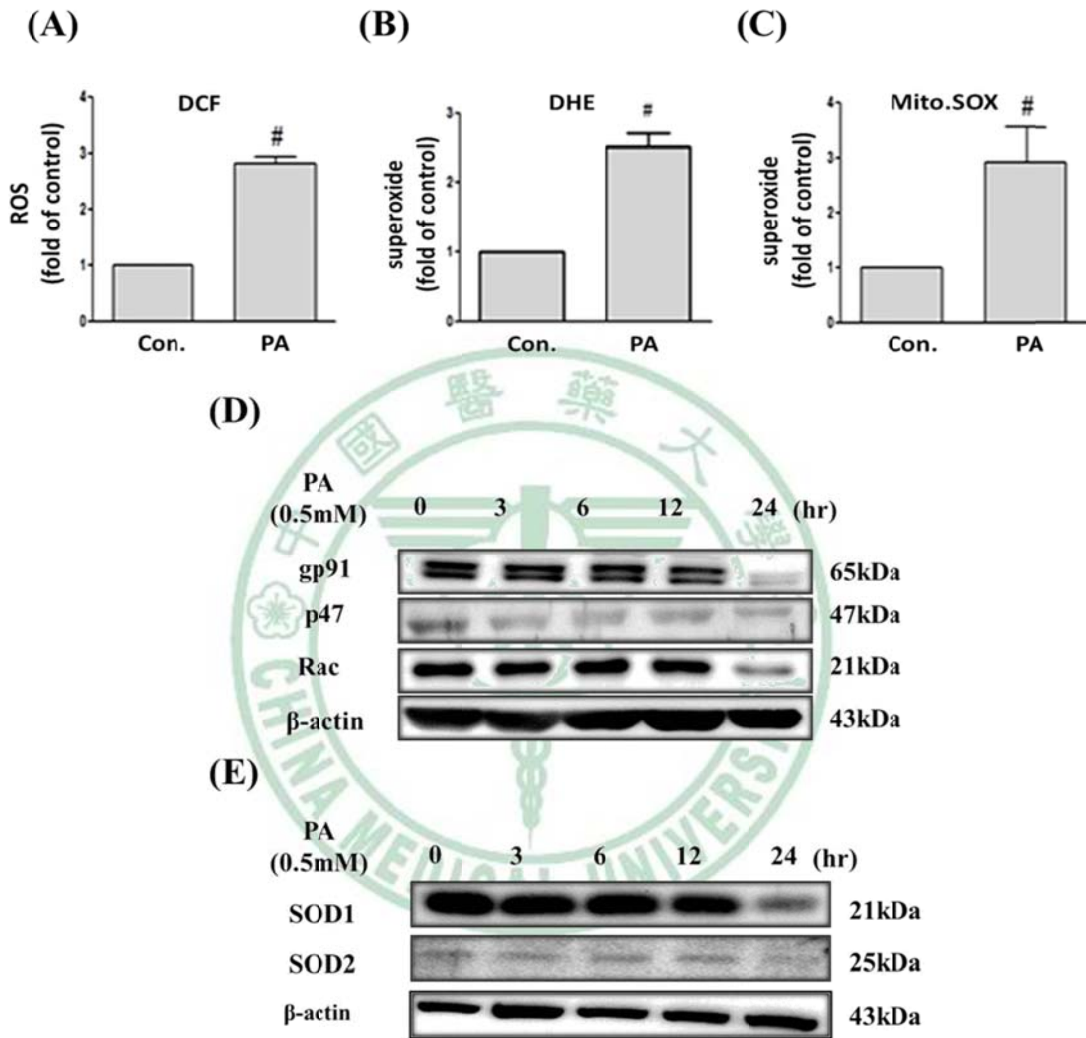
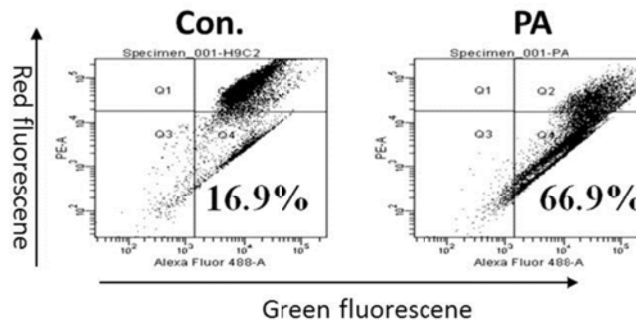


Fig.2 Effect of PA increased oxidative stress in H9c2 cells. H9c2 cells were treated with PA (0.5mM) for 24h followed by 1h incubation with fluorescent probe (A) DCF-AM (10μM) (B)DHE(10μM)(C) MitoSOX™ (5μM). Fluorescence intensity of cells was measured by flow cytometry. For Western blot analyzed, H9c2 cells were exposed to PA (0.5mM) for 0-24h. (D) The level of NADPH oxidase (Nox2-gp91, $p47^{phox}$, $p22^{phox}$, Rac) protein expression. (E) The level of antioxidant enzyme SOD1,

SOD2 protein expression. Data showed the means±SEM of 3 independent analyses.*$P<0.05$ vs. palmitic acid alone treatment.

Fig.3

(A)



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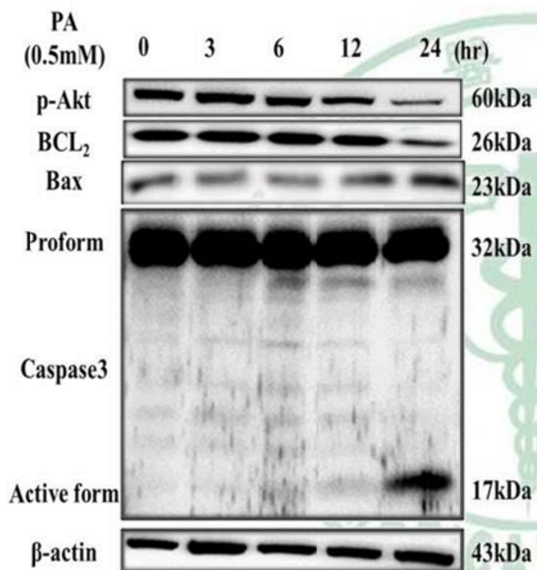

(C)

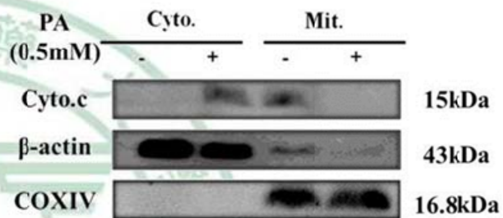

Fig.3 Palmitic acid leads to unstability of mitochondria member potential, lead to cell apoptosis through mitochondria dependent pathway. H9c2 cells were incubated with PA (0.5mM) for 24h. (A) $\Delta\Psi m$ was assessed with signal from monomeric and J-aggregate JC-1 fluorescence. Left control; right PA, JC-1 fluorescence was measured by flow cytometry. (B) The protein level were measured by Western blot after exposed PA (0.5mM) in 0-24h. Summary data show that PA down-regulated anti-apoptoic (BCL_2) protein and survival (p-AKT^{Ser473}) also increased pro-apoptotic (Bax) protein casepase3 activation. (C)For

Western blot analyses, after treated with PA (0.5mM) for 24h, cytochrome c were release from mitochondrial to cytosolic.

Fig.4

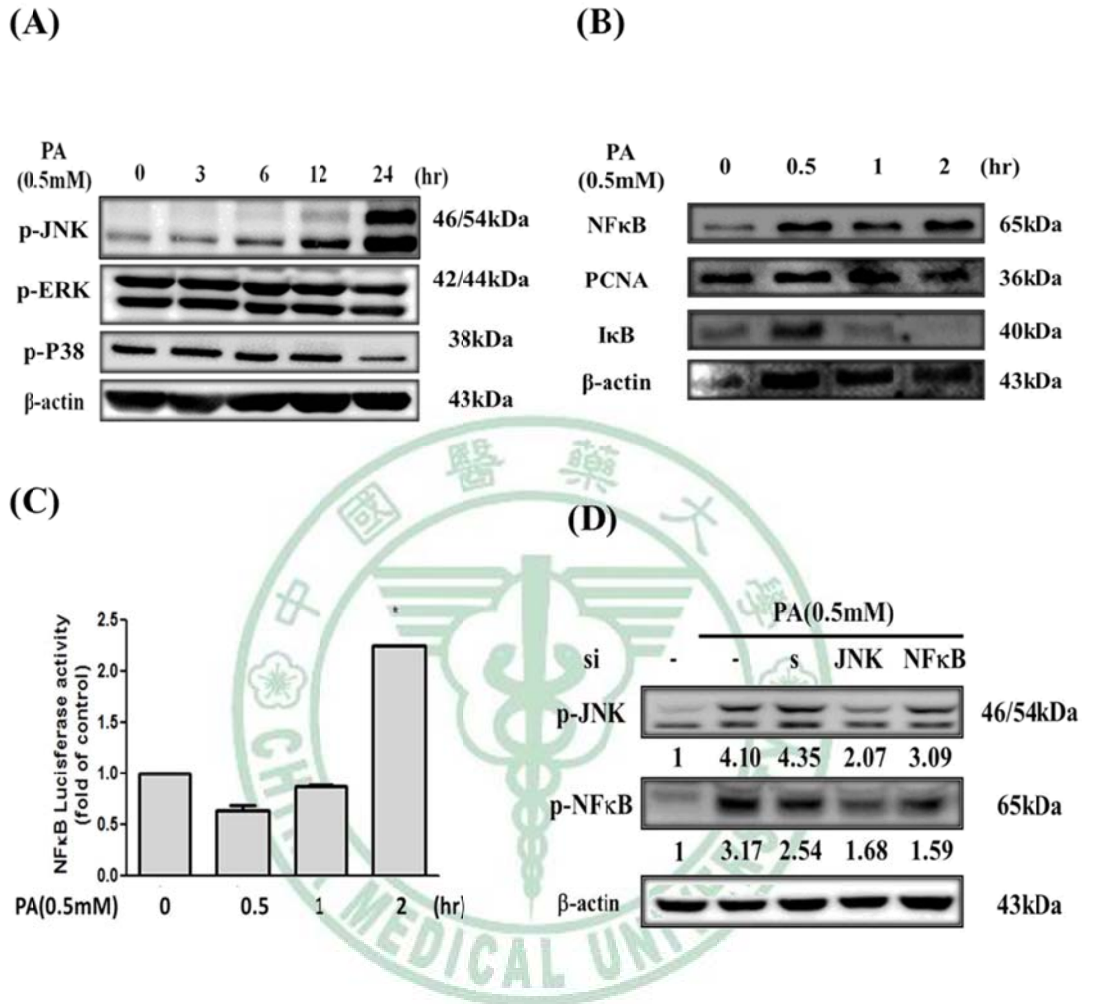

Fig.4 Effect of PA on activated of MAPK family proteins, NFκB signaling pathway in H9c2 cells. H9c2 cells were treated with PA (0.5mM) for various time periods (0-24h), and then were harvested and lysed. (A) Immunoblotted with antibodies against MAPK family (p-ERK, p-JNK, p-P38). H9c2 cells were incubated with PA (0.5mM) for 0-2h. (B) For western blot analyses, monoclone anti-IκB, NFκB, and anti-β-actin and anti-PCNA antibody were used for normalization of cytosolic and nuclear proteins were used. (C) NFκB luciferase reporter gene assay. (D)

After H9c2 cells were transfected with JNK1, NFκB siRNA (10nM) for 24h, followed by treatment of PA for 24h with or without NAC (500μM), scramble for non specific siRNA control. The levels of proteins indicated were analyzed by Western blot. Data showed the means±SEM of 3 independent analyses.*$P<0.05$ vs. palmitic acid alone treatment.

Fig.5

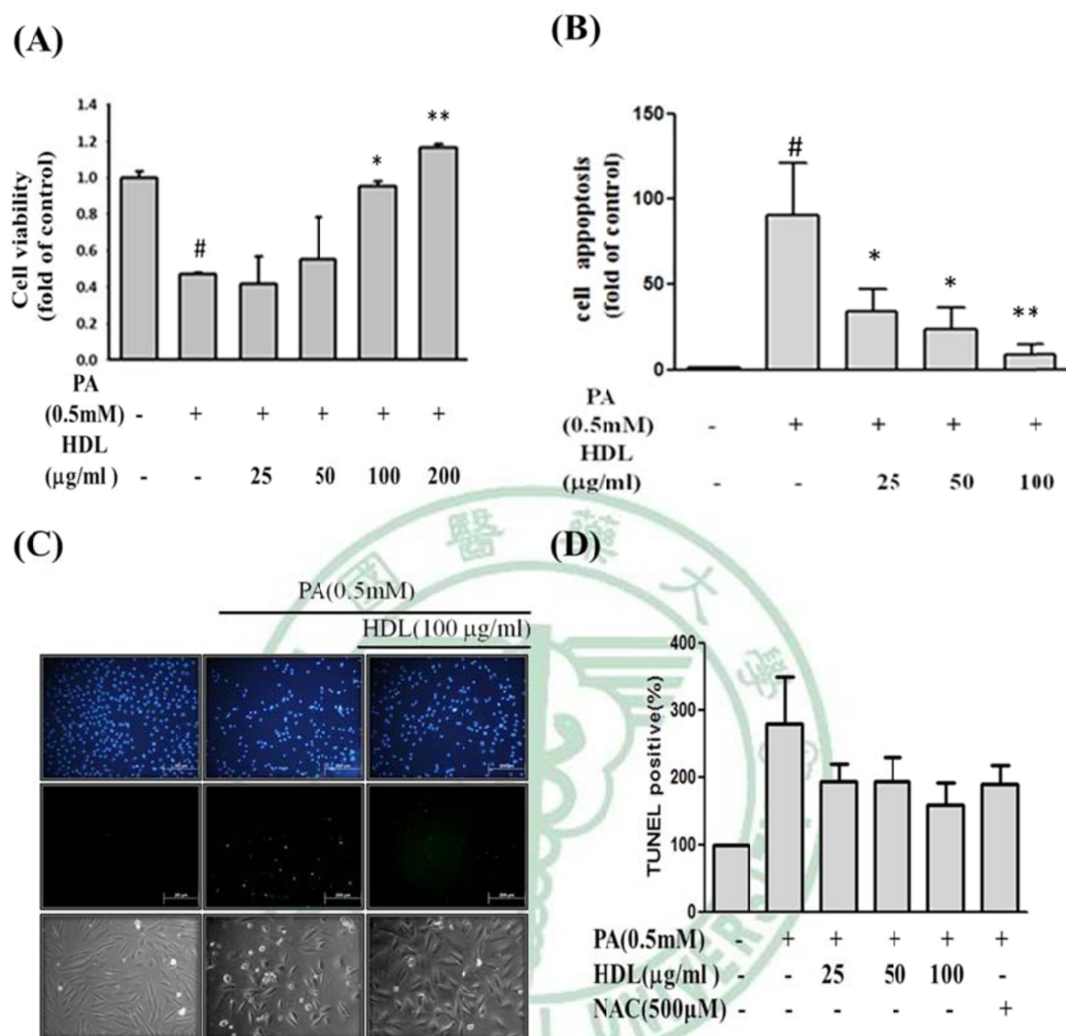

Fig.5 Effects of HDL on palmatic acid-induced H9C2 cells death. (A) H9c2 cells were incubated with PA (0.5mM) in the absence and presence of different concentrations of HDL (25-100μg/ml) for 24h, viability was determined via MTT assay. (B)Flow cytometry profile represents Annexin-V-FITC staining in *x* axis and PI in *y* axis. The number represents the percentage of early apoptotic cells in each condition. (C)Fluorescence images show the cells stained with 4,6-diamidino-2-phenylindole (DAPI) (upper panel) and stained using terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling

(TUNEL) assay (middle panel), and photomicrographs were from phase-contrast microscopy (bottom panel). (D) Flow cytometric analysis. Data showed the means±SEM of 3 independent analyses. #P < 0.01 vs. control; *P<0.05 vs. palmitic acid alone treatment.

Fig.6

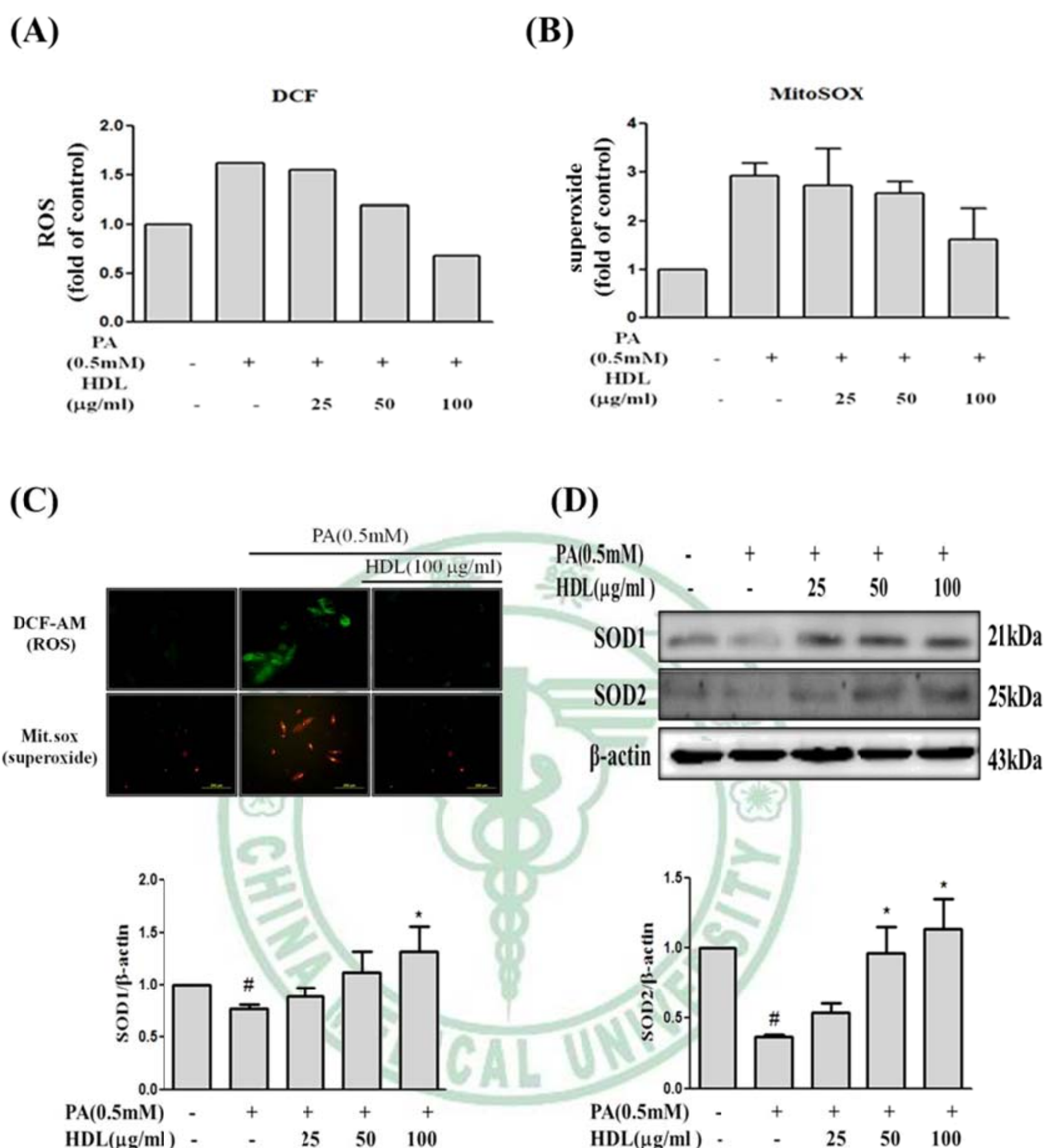

Fig.6 Inhibitory effect of HDL on palmitic acid induced ROS production in H9c2 cells. After pretreated for 2h with the indicated concentration of HDL (25-100μg/ml), 0.5mM PA were added to medium for 2h and followed by a 1h incubation with (A and C) DCF-AM (10μM), (B and C) MitoSOX™ (5μM). Fluorescence intensity of cells was measured by (A and B) flow cytometry and (C) phase-contrast microscopy (D) For Western blot analyses, monoclonal anti-SOD1,SOD2 and anti-β-actin, antibody (for normalization) were used. Data showed the

means±SEM of 3 independent analyses. #$P < 0.01$ vs. control ; *$P<0.05$ vs. palmitic acid alone treatment.

Fig.7

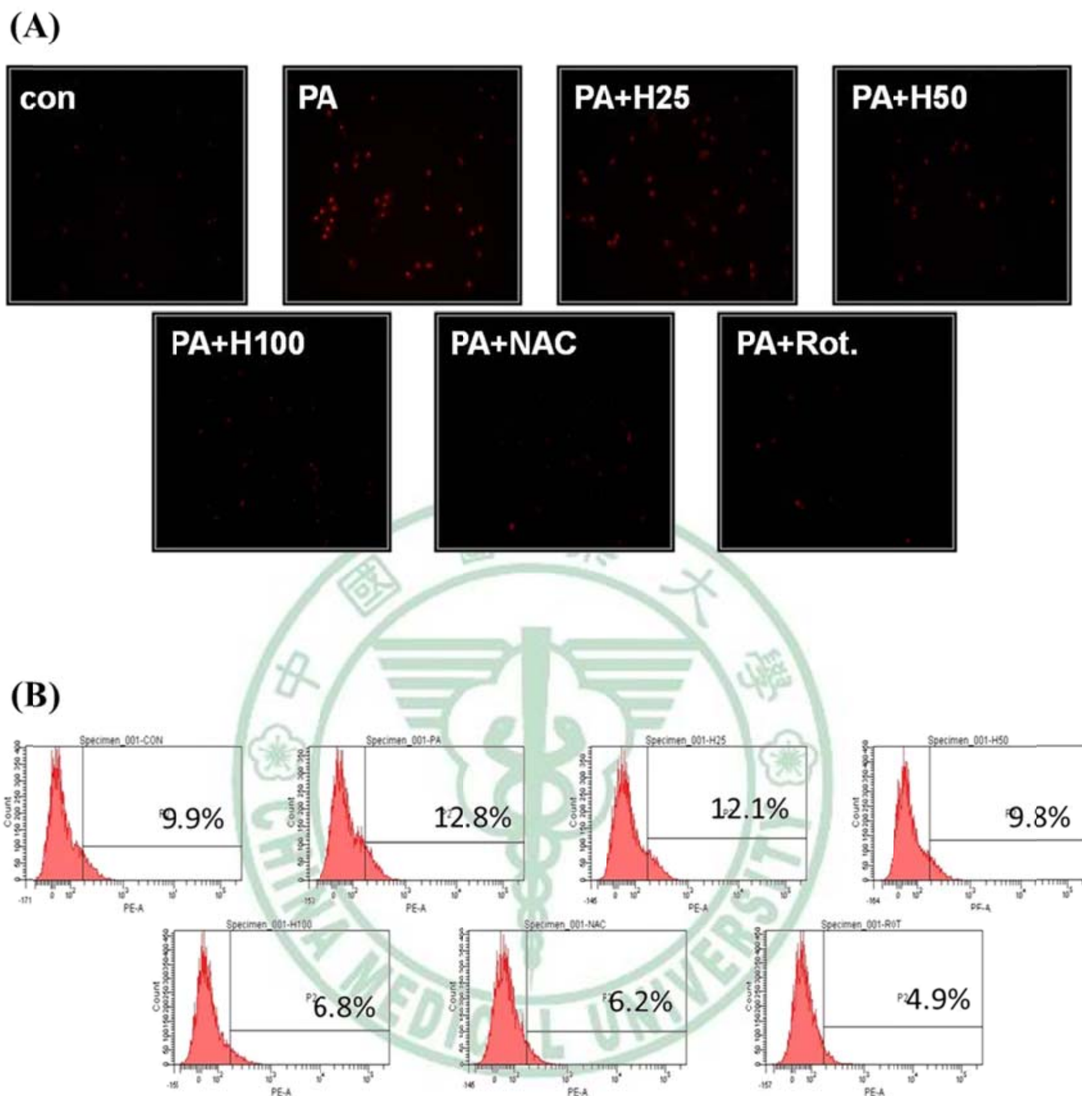

Fig.7 HDL can reduce mitochondrial ROS in neonatal cardiomyocytes treated with PA. Neonatal cardiomyocytes were treated with HDL 100μg/ml for 2h and then incubated with 0.5mM PA for an additional 24h, and followed by 1h incubation with MitoSOX™ (5μM). Fluorescence intensity of cells was measured by (A) phase-contrast microscopy and (B) flow cytometry.

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Fig.8

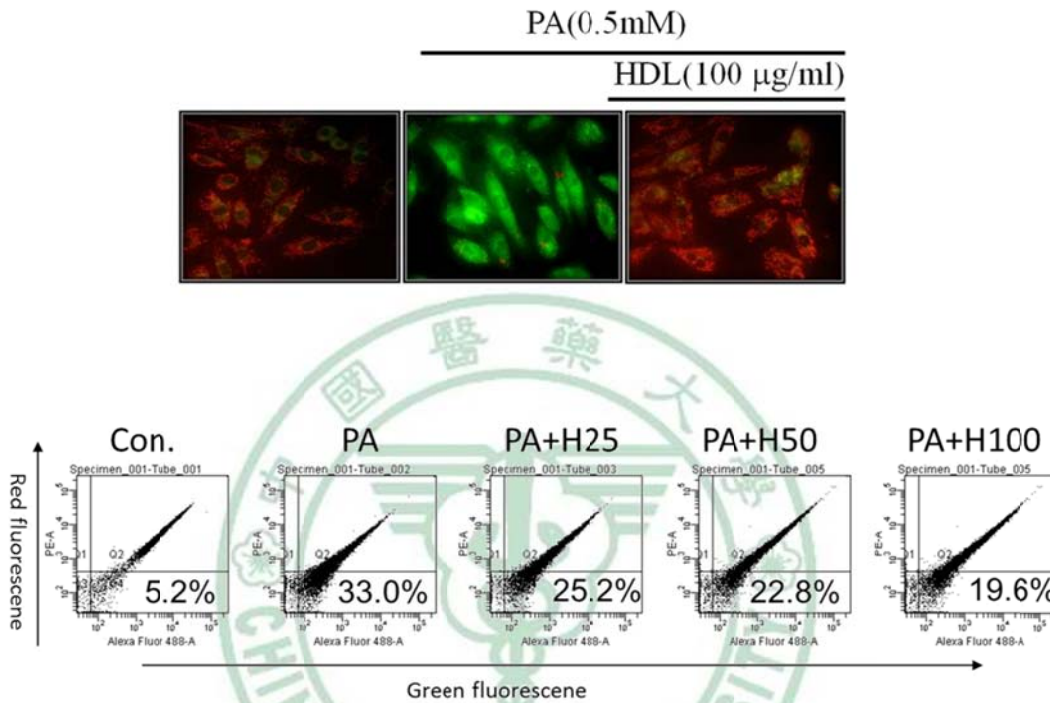


Fig.8 HDL stabilized on mitochondrial transmembrane permeability transition (ΔΨm) in PA-treated H9c2 cells. Cells were incubated with HDL 100μg/ml for 2h and then incubated with 0.5mM PA for an additional 24h. The change in mitochondrial membrane poteneial was assessed based on the signal intensity from monomeric (green) and J-aggregate(red) JC-1 fluorescence. No treatment (left); PA (middle); and PA + HDL (right).

Fig.9

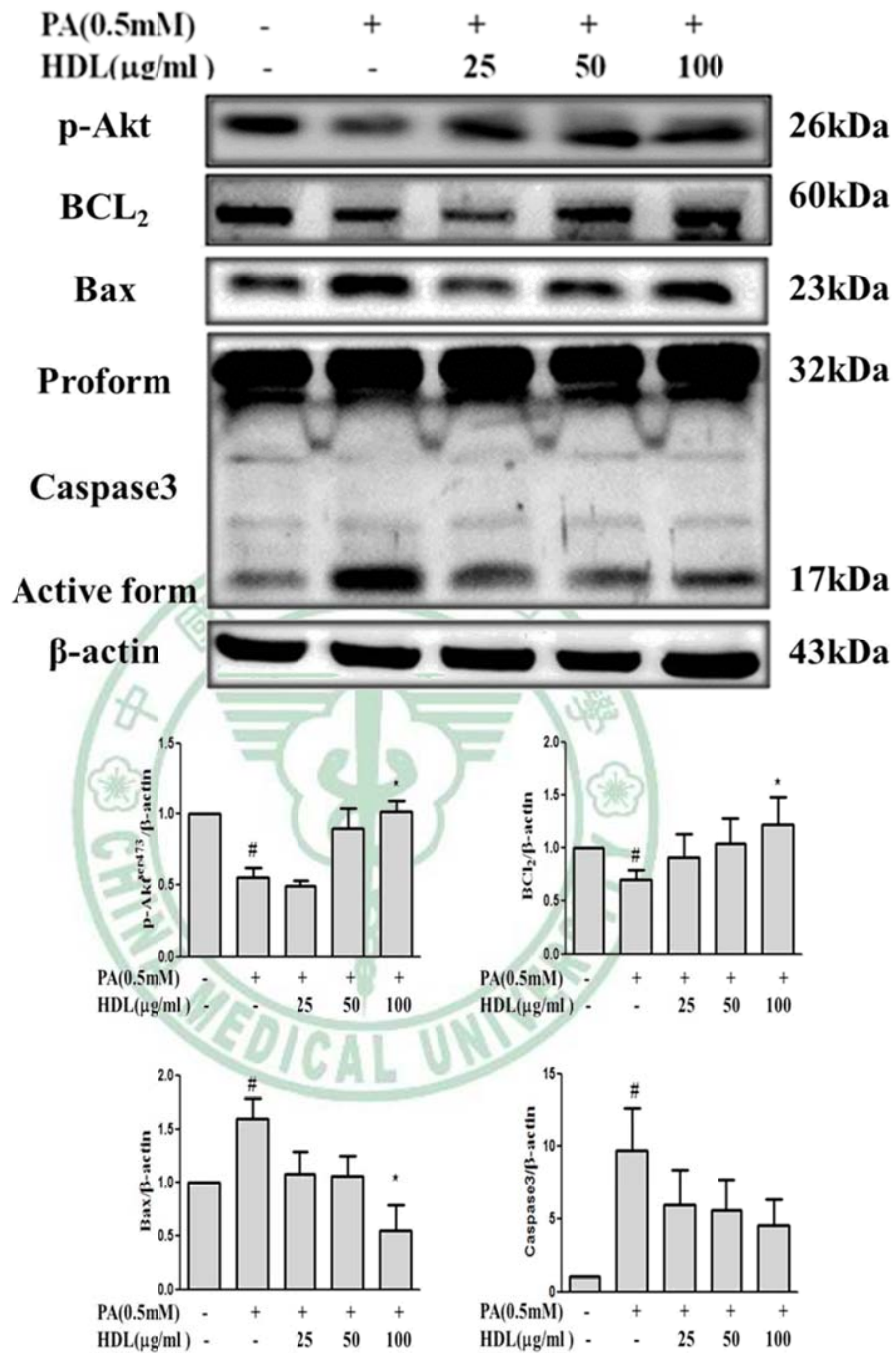


Fig.9 The effect of HDL on protein expression levels of mitochondrial dependent pathway in H9c2 cells. H9c2 cells were pretreated for 2h with the indicated concentrations of HDL (25-100μg/ml) followed by PA (0.5mM) for 24h. Representative Western blot and summary data show

that PA downregulated survival protein (p-AKT^{ser473}) and anti-apoptoic (BCL_2) protein and upregulated pro-apoptoic (Bax) protein and activate caspase3 protein expression, whereas HDL pretreatment suppressed these apoptosis-provoking alterations. Results were subjected to densitometric analysis. Data showed the means±SEM of 3 independent analyses. #P < 0.01 vs. control; *P<0.05 vs. palmitic acid alone treatment.

Fig.10

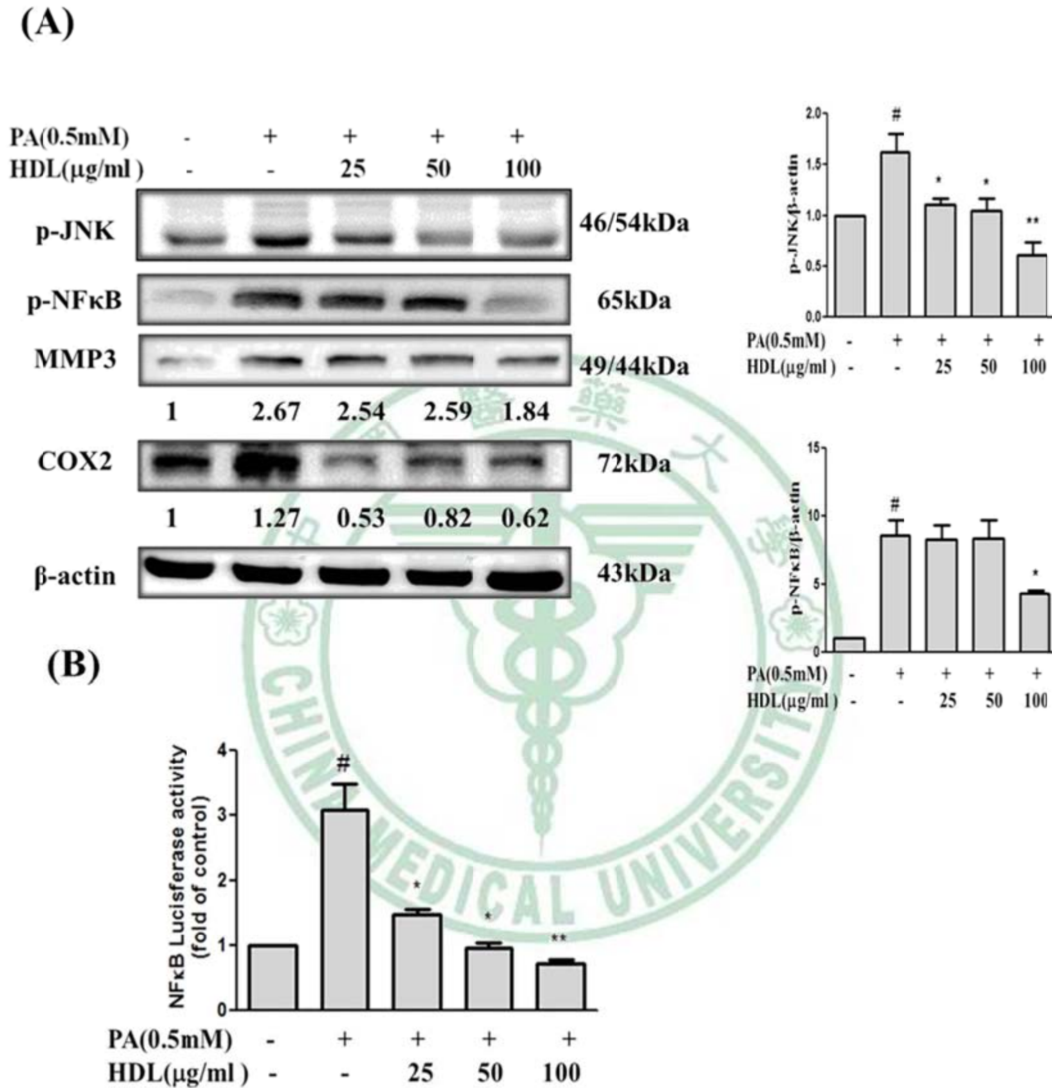
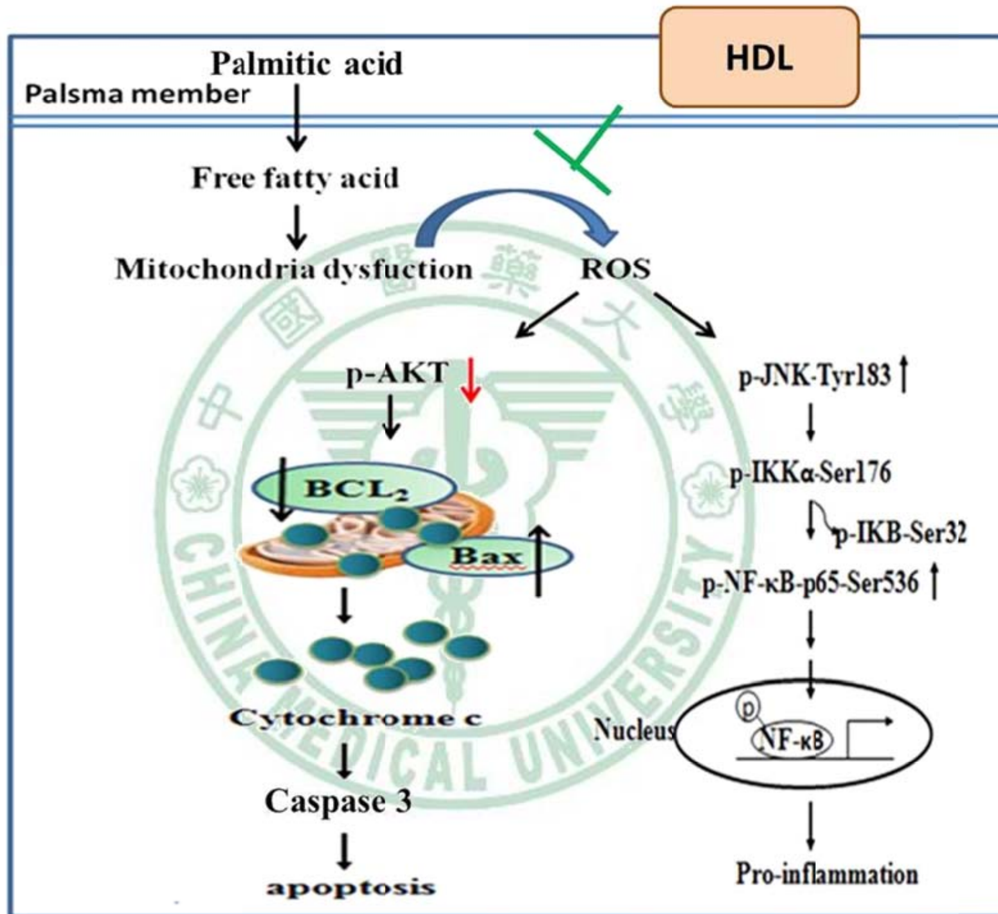


Fig.10 Effects of HDL on PA-triggered JNK phosphorylation and downstream protein NFκB activity in H9c2 cells. H9c2 cells were pretreated for 2h with the indicated concentrations of HDL (25-100μg/ml) followed by PA (0.5mM) for 24h. (A) For Western blot analyses, monoclonal anti-JNK, p-NFκB MMP3, cox_2 and anti-β-actin, antibody

(for normalization) were used. (B) NFκB Luciferase gene reporter assay. Data showed the means±SEM of 3 independent analyses. #P < 0.01 vs.control ; *P<0.05 vs. palmitic acid alone treatment.

Fig.11



第二部分
高密度脂蛋白保護缺氧對心肌細胞凋亡經由抑制 NADP 氧化酶相關的氧化壓力引起的訊息途徑

Part II
Protective effect of HDL on NADPH oxidase-derived super oxide anion mediates hypoxia-induced cardiomyocyte apoptosis

中文摘要

心臟疾病和心血管疾病為我國十大死因之一，而粥狀動脈硬化會導致心臟受損、中風、甚至死亡。動脈藉由血液提供氧氣給心臟，若有動脈斑塊的現象產生會造成冠狀動脈疾病，此現象會使心臟呈現缺氧的情形。研究中我們採用了老鼠心臟衍生的 H9c2 肌原母細胞以及新生鼠初代培養的心室細胞，給予長時間培養缺氧($1\%O_2$)的環境。結果顯示，在缺氧的環境下會導致氧化壓力的產生進而使心肌細胞凋亡及心臟功能喪失。本研究目的是利用缺氧模擬心肌梗塞的環境，觀察是否會因為高密度脂蛋白的保護，而減少缺氧對心肌細胞所造成的傷害和其相關分子機制。結果顯示，缺氧促進心肌細胞凋亡可能是透過活化NADPH 氧化酶而產生自由基，而其中 Angiotensin II receptor type 1 (AT1)和 PKC 或許會調控 NADPH 的活性。此外，缺氧會使存活蛋白 BCL_2 和 p-AKT^{ser473} 表現下降並且增加促凋亡蛋白 Bax 的表現。然而，前處理 HDL 兩個小時後發現可以有效的調控缺氧影響的分子作用機制。高密度脂蛋白明顯的抑制 NADPH 氧化酶的活化，同時也減少 AT1 和 PKC 的蛋白表現。除此之外，高密度脂蛋白增加存活蛋白並降低促凋亡蛋白的表現。由結果顯示，高密度脂蛋白有效抑制缺氧促進心肌細胞凋亡，其作用機制可能是透過抑制 AT1 和 PKC 調控的 NADPH 氧化酶，減少自由基產生進而減少心肌細胞造成的凋亡。

Abstract

Heart disease and cardiovascular disease are the top ten factors of death in Taiwan. Atherosclerosis can lead to serious problems, including heart attack, stroke, or even death. The arteries supply oxygen-rich blood from heart. Coronary heart disease (CHD) occurs if plaque builds up in the coronary arteries to cause the ischemic heart disease which will enhance myocardial remodeling and also induce myocardial hypoxia. Heart-derived H9c2 cells and neoeatal rat ventricular myocytes erer used in this study. H9c2 cells were treated with 1%O_2 for 24hr to induce reactive oxygen species (ROS), leading to cardiomyocyte apoptosis and cardiac dysfunction. Therefore, the aim of the present study is to test whether HDL could protect against hypoxia-induced cardiomyocyte injury and explore the possible mechanisms. We investigate the NADPH oxidase-produced ROS-related signalings and apoptosis in cardiomyocytes under hypoxia conditions, and it may be via AT1 and PKC activation. Furthermore, hypoxia downregulated the survival protein (p-AKT^{ser473}) and anti-apoptoic protein (BCL_2) expression, however, increased pro-apoptoic protein (Bax) expressions. These detrimental effects were ameliorated by HDL. Results from this study may provide insight into a possible molecular mechanism underlying HDL suppression of the hypoxia-induced cardiomyocyte dysfunction. Therefore, we elucidated the roles of the HDL on inhibiting hypoxia induced cardiomyocyte apoptosis is mediated through inhibiting NADPH oxidase-derived ROS and mitochondria depended apoptosis pathway.

Introduction

1. Hypoxia in heart diseases

Atherosclerosis, the top ten causes of death in Taiwan, is the term for the process of fatty substances, cholesterol, cellular waste products, calcium and fibrin (a clotting material in the blood) building up on the inner lining of an artery. The buildup is called plaque. Atherosclerosis is the usual cause of heart attacks, strokes, and peripheral vascular disease. Arteries are blood vessels that deliver blood from the heart throughout the body. Plaques generated in the heart's arteries can cause chest pain on exertion, and cut off the supply of oxygen. Sudden plaque rupture and clotting can cause cardiac cell death, which is called heart attack, or myocardial infarction.

The mammalian heart is an obligate aerobic organ. Under normoxic condition, the working heart produces an abundant supply of ATP, mainly from fat oxidation. Mammalian heart cells cannot produce enough energy under anaerobic conditions to maintain essential cellular processes; thus, a constant supply of oxygen is indispensable to maintain cardiac function and viability (Giordano 2005). Hypoxic/ischaemic injury is another major stress to the heart, leading to cardiomyocyte damage through activating fetal cardiac gene expression and specific signal transduction pathways (Chiu, Wang et al. 2010). Gene expression is adjusted to oxygen availability in the heart by several mechanisms, including regulation of gene transcription by the hypoxia-inducible basic helix-loop-helix transcription factor, hypoxia-inducible factor 1α (HIF-1α) (Giordano and Johnson 2001; Huang, Hickey et al. 2004). HIF-1α regulates the

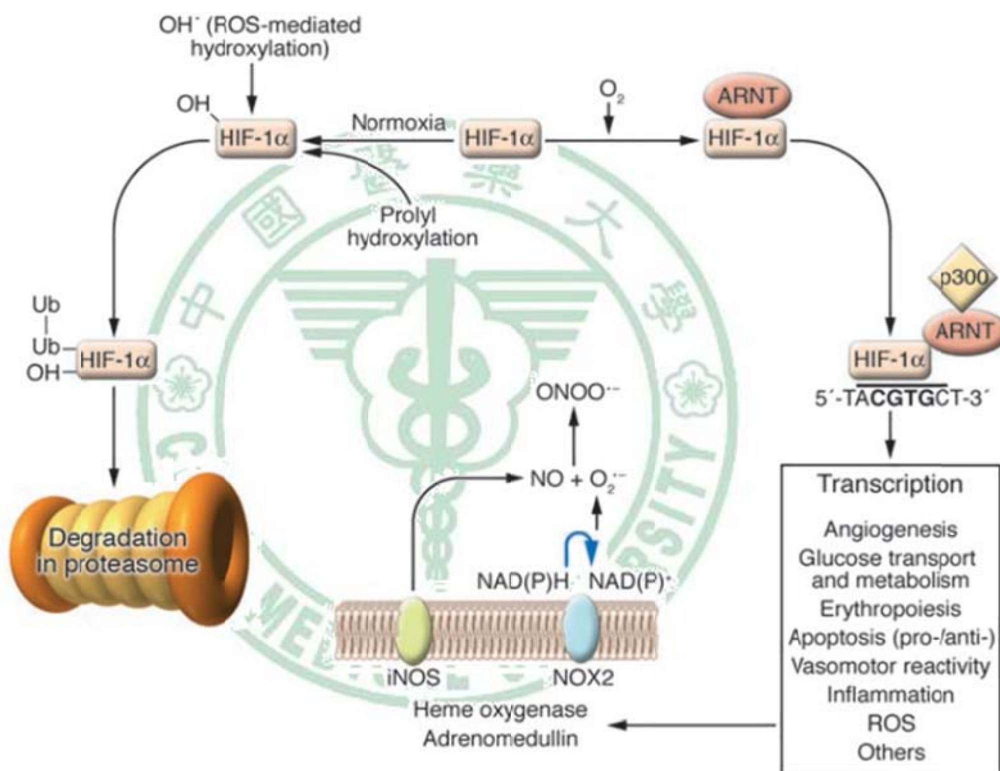
transcription of an extensive repertoire of genes, many of which can control angiogenesis and vascular remodeling, erythropoiesis, metabolism, apoptosis, control of ROS, vasomotor reactivity and vascular tone, and inflammation (Carmeliet, Dor et al. 1998; Iyer, Kotch et al. 1998; Ryan, Lo et al. 1998).

2. Hypoxia-induced oxidative stress in cardiomyocyte

Oxidative stress is caused by reactive oxygen species (ROS), also termed oxygen free radicals, which are molecules containing unpaired electrons and is derived from many cellular enzyme systems within the cardiovascular system (Fearon and Faux 2009). ROS is involved in inflammation, endothelial dysfunction, cell proliferation, migration and activation, extracellular matrix deposition, fibrosis, angiogenesis, and cardiovascular remodeling. These are important processes contributing to cardiovascular and renal remodeling in hypertension, atherosclerosis, diabetes, cardiac failure, and myocardial ischemia-reperfusion injury (Pawlak, Naumnik et al. 2004; San Martin, Du et al. 2007).

A significant number of in vitro and animal studies have demonstrated ROS is largely produced in the cardiovascular system in response to various stressors and in the failing heart(Ide, Tsutsui et al. 1999; Sawyer, Siwik et al. 2002; Sabri, Hughie et al. 2003; Suematsu, Tsutsui et al. 2003). Coronary artery disease (CAD) with consequent myocardial ischemia and necrosis is a leading cause of heart failure worldwide. It is important to note that ROS may play an important role in the genesis and progression of CAD (Pennathur, Wagner et al. 2001; Khatri, Johnson et al. 2004). Under hpoxia condition, HIF-1α regulates the transcription of an

extensive repertoire of genes, many of which can control angiogenesis and vascular remodeling, erythropoiesis, metabolism, apoptosis, control of ROS, vasomotor reactivity and vascular tone, and inflammation (Carmeliet, Dor et al. 1998; Iyer, Kotch et al. 1998; Ryan, Lo et al. 1998). In APP.1 showed the mechanisms by which HIF levels are regulated by oxygen and the maner in which ROS and HIF may interact.



APP.1 Transcriptional gene regulation by the hypoxia-inducible factor HIF-1

HIF-1α protein undergoes rapid prolyl hydroxylation under normoxic conditions by specific cellular prolyl hydroxylases. Direct hydroxylation by ROS is a alternative pathway. Hydroxylated HIF will interact with the VHL, a critical member of an E3 ubiquitin ligase complex that

polyubiquitylates HIF (Ub, ubiquitin), and targets HIF-1α for destruction by the proteosome. Under hypoxia (↓O2), hydroxylation does not occur and HIF-1α is stabilized. Heterodimerization with ARNT forms the active HIF complex that binds to a core hypoxia response element in a wide array of genes involved in a diversity of biological processes germane to cardiovascular function. Transcriptional activation of iNOS expression is shown as an example of how HIF-mediated gene expression can affect ROS generation by generating NO that interacts with $O2^{\bullet-}$ to form $ONOO^{\bullet-}$. NOX2 is shown as a cellular source of $O2^{\bullet-}$(Giordano 2005).

ROS can be formed in the heart by a variety of mechanisms, including generation during oxidative phosphorylation in the mitochondria as a byproduct of normal cellular aerobic metabolism (Davies 1995; Ide, Tsutsui et al. 1999). In the heart, mitochondria are needed to activate hypoxia-responsive pathways, which help restore O_2 levels and are jump started by the stabilization of hypoxia-inducible factor (HIF)-1α. By uncoupling mitochondrial O_2 consumption from ROS production, it is identified that the ROS are the key. These cells could not respire or make ATP, but they still can produce ROS and respond to hypoxia by stabilizing HIF-1α (Bell, Klimova et al. 2007).

3. Hypoxia-induced cardiomyocyte apoptosis

Cardiomyocytes seem to be more sensitive to hypoxia and ischemia than other cell types (Takemura et al., 2004). Hypoxia elicits a variety of functional responses in cardiomyocytes, including cell proliferation, cell hypertrophy and cell death. Cardiomyocytes respond to hypoxia to maintain homoeostasis by expressing a number of genes, which are

induced by a variety of signalling cascades (Wang, Chang et al. 2001; Wang, Li et al. 2002; Miano 2003; Zhang, Azhar et al. 2004). Apoptosis is usually induced by physiological stimuli, but can also be induced pathologically. It requires energy in the form of ATP, and gene transcription and protein synthesis are also involved. Myocardial ischemia leads to cardiac cell loss and scar formation, resulting in reduced pumping capacity which eventually leads to congestive heart failure and death(Pfeffer and Braunwald 1990). Hypoxia alone is sufficient to indue apoptosis in primary cultures of neonatal and cardic myocytes(Long, Boluyt et al. 1997). Hypoxia-induced apoptosis of cardiomyocytes is associated with activation of the mitochondrial dependent apoptotic pathway, which is regulated by the Bcl family by decreases in the anti-apoptotic protein and increasing in pro-apoptotic protein (Azhar, Liu et al. 1999; Kubasiak, Hernandez et al. 2002; Kubli, Ycaza et al. 2007) .

4. Hypoxia induced Angiotensin II expression

Inducted Angiotensin II attenuates chemical hypoxia-induced caspase-3 activation in primary cortical neuronal cultures. Some studies showed that inhibition of the renin–angiotensin cascade, by angiotensin-converting enzyme (ACE) inhibitor (Goll, Nyhan et al. 1986; Nyhan, Chen et al. 1992; Nong, Stassen et al. 1996) or angiotensin II receptor blockade (Kiely, Cargill et al. 1995; Kiely, Cargill et al. 1996; Zhao, al-Tubuly et al. 1996), reduces pulmonary vascular tone in normoxia (Goll, Nyhan et al. 1986; Nyhan, Chen et al. 1992) and hypoxia (Kiely, Cargill et al. 1995; Morrell, Atochina et al. 1995; Zhao, al-Tubuly et al. 1996). In the present

study, the effect of hypoxia on the expression of AngII on the induction of myocardin expression and gene transcription in neonatal cardiomyocytes and the specific signal transduction pathway mediating myocardin expression under hypoxia, and the hypoxic injury to cardiomyocytes also stimulates the expression of AngII, similar to other mechanical loads (Chiu, Wang et al. 2010).

5. High density lipoprotein (HDL)

HDL is a complex, bioactive particle, containing multiple acute phase response proteins, protease inhibitors, and complement regulatory proteins. The functionality of HDL can be influenced by abundance of various bioactive proteins and lipids, and can exert anti-inflammatory, anti-oxidative, anti-coagulative and other atheroprotective functions (Vaisar, Mayer et al. 2010).

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Materials

40%Acrylamide/Bis solution 29:1 (SERVA, Germany)

Ammonium persulfate/APS (USB, USA)

beta-mercaptoethanol (Pharmacia Biotech, Sweden)

Bovine serum albumin/BSA (Sigma, USA)

Bromophenol Blue (Sigma, USA)

Cosmic Calf Serum/CCS (Hyclone, USA)

DAPI (Sigma, USA)

DCF-AM (2',7'-dichlorofluorescein acetoxymethyl ester; Molecular Probes, Eugene, OR)

Dihydroethidium(DHE)

DMEM (Dulbeccco's Modified Eagle's Medium; Sigma, USA)

DMSO (dimethyl sulfoxide; Sigma, USA)

DTT (1,4-Dithio-D, L-threitol; GERBU, Germany)

Ethylenediaminetetraacetic acid/EDTA (Sigma, USA)

FBS (Fetal bovine serum; GIBCO, USA)

Glucose (USB, USA)

Glycine (Sigma, USA)

Glycerol (Amresco, USA)

Mem-PER®Eukaryotic Membrane Protein Extraction Reagent Kit (PIERCE, 89826)

Methanol 20L (慕容科技有限公司/Taiwan)

Neonatal Rat Cardiomyocyte Isolation Kit (Cellutron Life Technology, MD, USA)

Paraformaldehyde(Sigma, USA)

PBS (GIBCO, New Zealand)

Penicillin (Sigma, USA)

Protease inhibitor cocktail tablets (Roche, Germany)

Protein maker (Fermentas)

PVDF membrane pore size 0.45 m (Millipore, USA)

Sodium dodecyl sulfate /SDS (Sigma, USA)

Sodium chloride/NaCl (Sigma, USA)

Sodium bicarbonate/$NaHCO_3$ (Sigma, USA)

TEMED (Sigma, USA)

Tris(USB, USA)

Tris-base (USB, USA)

Tris-HCl (USB, USA)

Triton X-100(TEDIA, USA)

TUNEL (Terminal deoxynucleotide dUTP-biotin nick-end labeling; Roche, Mannheim, Germany)

Tween 20(Pharmacia, Sweden)

Trypsin-EDTA (GIBCO, USA)

脫脂奶粉(安佳, New Zealand)

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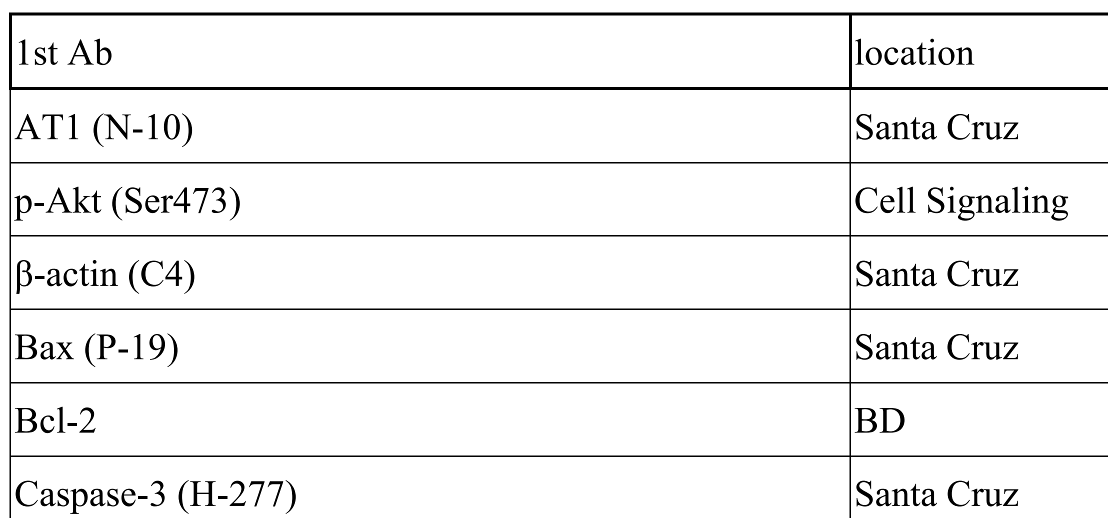

1st Ab	location
AT1 (N-10)	Santa Cruz
p-Akt (Ser473)	Cell Signaling
β-actin (C4)	Santa Cruz
Bax (P-19)	Santa Cruz
Bcl-2	BD
Caspase-3 (H-277)	Santa Cruz

ERK1	BD
p-SAPK/JNK (Thr183/Tyr185)	Cell Signaling
NOX-2/gp91 phox	abcam
p22-phox (FL-195)	Santa Cruz
p47-phox	upstate
p-p38 (D-8)	Santa Cruz
p-PKCα(Ser657)	upstate
p-PKCδ(Thr505)	Cell Signaling
Rac1	abcam
SOD-1 (C-17)	Santa Cruz
SOD-2 (MnS-1)	Santa Cruz
2st Ab	location
anti-mouse IgGhorseradish peroxidase conjugated	Santa Cruz
anti-rabbit IgGhorseradish peroxidase conjugated	Santa Cruz
anti-gout IgGhorseradish peroxidase conjugated	Santa Cruz

Methods

1. Cell culture

H9c2 cell lines were obtained from American Type Culture Collection (ATCC), cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% Cosmic CalfR serum (CCS), 2mM glutamine, 100units/ml penicillin, 100μg/ml streptomycin, and 1mM pyruvate in humidified air (5% CO2) at 37 ^{o}C. During the treatment, pretreated with HDL for 2 hours and then stimulated in 1%hypoxia for 24 hours. The specificity of the inhibit ROS and mitochondria complex Ⅰ inhibitor by adding N-acetly cysteine(NAC) (500μM).

2. Reactive oxygen species and mitochondrial superoxide production

Intracellular ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes), dihydroethidium (DHE) and MitoSOX™ as a probe for the presence of H_2O_2 or superoxide. DCFH-DA is converted by intracellular esterases to DCFH, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant, and then analyzed by flow cytometry. MitoSOX™ Red mitochondrial superoxide indicator is a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells, which is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence. MitoSOX™

is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species (RNS)–generating systems, and oxidation of the probe is prevented by superoxide dismutase. The oxidation product becomes highly fluorescent upon binding to nucleic acids.

3. Immunoblotting

Culture H9c2 cells were scraped and washed once with PBS, then cell suspension was spun doen, and lysed in RIPA buffer (HEPES 20mM, MgCl2 1.5mM, EDTA 2mM, EGTA 5mM, dithiothreitol 0.1mM, phenylmethylsulfonyl fluoride 0.1mM, pH 7.5), and spun down 12,000 rpm for 20 min, the supernatant was collected in new eppendorf tube. Proteins (30 μg) were separated by electrophoresis on SDS-polyacrylamide gel. After the protein had been transferred to polyvinylidene difluoride membrane, the blots was incubated with blocking buffer (1X PBS and 5% nonfat dry milk) for 1 hour at room temperature and then probed with primary antibodies (1:1000 dilutions) overnight at 4°C , followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody at a 1:50000 dilution. The bound immunoproteins were detected by an ECL kit.

4. DAPI staining and TUNEL assay

After various treatments, H9c2 cells grown on 6 mm plate were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with PBS, cells were treated with permeation solution

(0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4ºC. Following wash with PBS, samples were first incubated with TUNEL reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate-dUTP. The cells were also stained with 1μg/ml DAPI for 30 min to detect cell nucleus by UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope, respectively. Apoptotic cells were assessed by fluorescence microscope or in a flowcytometer.

5. Cardiomyocyte Culture

Neonatal cardiomyocytes were isolated and cultured using the commercial Neonatal Cardiomyocyte Isolation System Kit according to manufacturer's directions. Briefly, hearts from one- to two-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion solution at 37 °C. Ventricular cardiomyocytes were isolated and cultured in DMEM containing 10% fetal bovine serum, 100μg/ml penicillin, 100μg/ml streptomycin, and 2 mM glutamine. After 3-4 days, cells were incubated in serum-free essential medium overnight before treatment with indicated agents.

6. Statistical analysis

Statistical differences were assessed by one way-ANOVA. $P < 0.05$ was considered statistically significant. Data were expressed as the mean ± SEM.

Results

Sustained incubation of H9c2 cells in hypoxia conditions increases reactive oxygen species (ROS) production and decreases antioxidant enzyme expression

Cardiovascular injury, one of the most common hypoxia complications,is linked to the elevated ROS levels, which subsequently induce cell apoptosis. Therefore, we examined the cellular ROS levels in cardiomyoblast H9c2 cells incubated in sustained hypoxia conditions. The $gp91^{phox}$ is the catalytic core of this complex and $p22^{phox}$ is the only other membrane component of the vascular NADPH oxidases. On stimulation, $p47^{phox}$ becomes phosphorylated and forms a complex and translocates to the membrane, where it is associated with $gp91^{phox}$ and $p22^{phox}$ to assemble the active oxidase (Zeng, Han et al. 2010). The effects of hypoxia on $gp91^{phox}$ and $p22^{phox}$ expression and membrane translocation of $p47^{phox}$ and Rac-1 were examined by Western blotting in H9c2 cells with membrane and cytosolic isolation. We found that the protein levels of NADPH oxidases were increased significantly in H9c2 cells exposed to hypoxia for 0-24 h(Fig.1A). There is growing evidence indicated that AT1 and PKC induced by hypoxia have been shown to contribute to the activation of NADPH oxidase.

As show in Fig.1B, after treatement with hypoxia for different time periods, the protein levels of AT1, and phosphorylation of PKCα and δ significantly increased in H9c2 cells. Intracellular ROS levels are regulated by the balance between ROS generation and activity of antioxidant enzymes such as catalase or SOD. Thus, the involved ROS is

able to inactivate antioxidative enzymes that additionally increase the imbalance in favor of oxidative stress. Therefore, we investigated the expression of its isoforms in H9c2 cells in response to hypoxia. Our results showed that the antioxidant enzymes SOD2 decreased in H9c2 cells treated with hypoxia (Fig.1B).

Hypoxia-induced apoptosis in cardiomyocyte

To clarify hypoxia induced cell apoptosis in cardiac myocyte, we performed TUNEL analysis for observing cells undergoing apoptosis. After incubation with hypoxia for 24h, we observed a significant increase apoptosis bodies (Fig.2A). Next, we examined the survival protein (p-AKT^{ser473}) in different time of hypoxia condition. The results showed hypoxia downregulated the survival protein expression (Fig.2B).

Roles of MAPK family proteins in hypoxia induced H9c2 cell apoptosis

To investigate whether MAPK family protiens are involved in the hypoxia-induced H9c2 apoptosis, we examined the expression levels of MAPK family proteins by Western blot. Our results showed that the phosphorylation of ERK, JNK, and P38 were increased after treatment with hypoxia for 0-24h (Fig.2C).

Effects of HDL on hypoxa-induced NADPH oxidase complex and antioxidant enzyme protein expression

The functionality of HDL can be influenced by the abundance of various bioactive proteins and lipids, that exert anti-inflammatory, anti-oxidative,

anti-coagulative and other atheroprotective functions(Vaisar, Mayer et al. 2010).So, we would like to know whether HDL can downregulated the effect of hypoxia in H9c2 cardiomyocyte cells.

The effects of HDL (25-100μg/ml) on $gp91^{phox}$ and $p22^{phox}$ expressions and membrane translocation of $p47^{phox}$ and Rac-1 were examined by Western blotting in H9c2 cells with membrane and cytosolic isolation. We found hypoxia treatment of H9c2 cells resulted in the increase of NADPH oxidase activity. However, pretreatment of hypoxia-exposed cells with HDL (25-100μg/ml) led to a does-dependent reductions in $gp91^{phox}$ and Rac-1 protein expression (Fig.3A). As shown in Fig.3A, incubation of H9c2 cells with hypoxia resulted in significant phophorylation of protein kinase C and HDL attenuated this protein activation. Our result also showed that HDL (25-100μg/ml) reversed the reduced of SOD activity caused by hypoxia. In order to explore whether HDL influence ROS and superoxidase generation in H9c2 cells, we investigated the effects of HDL on generation of ROS, a potential factor related to hypoxia-induced H9c2 cells injury, by using hydroxyl radical sensitive probe 2’,7’-dichlorofluorescein acetoxymethyl ester (DCF-AM), superoxide sensitive probe dihydroethidium (DHE) and MitoSOX™ Red mitochondrial superoxide indicator. The levels of ROS and superoxide generations examined by flow cytometry have significantly decreased in H9c2 cells with pretreatment of HDL (25-100μg/ml) for 2 h before exposure to hypoxia in a dose-dependent manner (Fig.3B).

Sustain exposure of HDL can reduce NADPH oxidase activity in neonatal cardiomyocytes treated with hypoxia.

In order to explore whether HDL influence NADPH oxidase, a major source of ROS in H9c2 cells, we measured the effects of HDL on the formation of superoxide by using superoxide sensitive probe dihydroethidium (DHE). As shown in Fig.3C, neonatal cardiomyocytes treated with HDL (25-100μg/ml) for 2h before exposure to hypoxia for 24h, we used superoxide sensitive probe dihydroethidium (DHE) to confirm by microscopic observation. The resuls showed HDL (25-100μg/ml) enhanced superoxide generation to reduced induced by hypoxia to control similar results were obsereved by the treatments of anti-oxidant (NAC,500μM) and mitochondrial superoxide inhibitor (Rotenone,5μM).

Effect of HDL on hypoxia-induced MAPK protein phosphorylations

Incubation of H9c2 cells with hypoxia resulted in significant phophorylation of p38MAPK, JNK and ERK. Therefore, we would like to examine whether HDL can inhibit MAPK protein activation. H9c2 cells were treated with HDL (25-100μg/ml) for 2h before exposure to hypoxia for 24h, it data showed HDL can downregulate the phophorylations of ERK, JNK, p38 proteins (Fig.4A).

HDL attenuated the apoptoic effects of hypoxia by regulating Bcl2 family protein, and activation of casepase3

Whether HDL can influence apoptosis related proteins was investigated. Immunoblotting studies demonstrated that hypoxia

downregulated the antiapoptotic and survival protein (BCL_2, p-AKT^{ser473}), also upregulated the proapoptoic protein (Bax), whereas HDL treatment effectively repressed these hypoxia-induced proapoptoic events (Fig.4B). Since activated caspase 3 is a key factor in the execution of mithchondrial apoptosis (Narula, Pandey et al. 1999), Whether HDL ultimately influence this factor to modulate apoptosis, we subsequently determined its pro-form and active-form by immunoblotting (Fig.4B). The data showed that active caspase 3 was significantly increased in cells that had been treated with hypoxia was suppressed in cells induced by hypoxia was suppressed by the treatment of HDL. To further confirm whether the antiapoptic effects of HDL on hypoxia-induced cell death, TUNEL and DAPI staining assays were performed and evaluated by microscopic observation. As shown in Fig.4B, cells incubated with 1%hypoxia for 24h showed typical features of apoptosis, including the formation of condensed nuclei, which were, however, not observed in the HDL-pretreated H9c2 cells. As described above, both results of cell viability assay and phenotypic observation of apoptosis under microscopy suggested that HDL is a potent inhibitor of hypoxia-induced cytotoxicity in cultured H9c2 cells. NAC (500μM) could suppress increased TUNEL-positive cell number induced by hypoxia.

Discussion

Hypoxic condition is a general cause of cell damage, which is implicated in many pathologic conditions including stroke, myocardial infarction (MI), diabetes mellitus, multiple organ failure (Halterman and Federoff 1999; Kim, Ahn et al. 2004). Plaques formation in the heart's arteries will leading to myocardial remodeling and myocardial hypoxia (Lee, Wolf et al. 2000). It has reported that induced Angiotensin II attenuates chemical hypoxia-induced caspase-3 activation in primary cortical neuronal cultures (Chiu, Wang et al. 2010). However, in hypoxia condition, we found hypoxia downregulated the antiapoptoic proteins (BCL_2 and p-AKT^{ser473}) and upregulated the proapoptoic (Bax) proteins. Furthermore, increased caspase 3 activity in H9c2 cells with hypoxia (Fig.2B) can be decreased by treatment of HDL (Fig.4).

We want to investigate whether production of Angiotensin II lead to generate hypoxia-induced ROS.In this study, we explore the mechanisms of hypoxia-induced cell apoptosis in heart, focusing on the NADPH oxidase-generated ROS induced signaling. This study also extends the therapeutic potenial of inhibiting NADPH oxidase in hypoxia-exposed cardiac cells. The effects of ang II are mediated by two receptors, referred to as the Ang II type-1 (AT1) and type-2 (AT2) receptor subtypes. AT1 receptor is dependend on the cell and organ type, stimulation of these signal transduction pathways leads to cellular contraction, hypertrophy, proliferation, and/or apoptosis. One of the most important effects of AT1-receptor activation, particularly in the cardiovascular system, is the production and release of reactive oxygen species (ROS)(Nickenig and

Harrison 2002). The effect of hypoxia resulted in increased AT1 and NADPH oxidase protein expression (Fig.1). Various protein kinases have been involved in the regulation of NADPH oxidase activity (McPhail, Qualliotine-Mann et al. 1995; El Benna, Faust et al. 1996), among which the protein kinase C (PKC) family appears to play a major role (Wolfson, McPhail et al. 1985; Nauseef, Volpp et al. 1991; Combadiere, el Benna et al. 1993). As show in Fig.1B, hypoxia increased phosphorylation of PKC α and δ. Furthermore, we suggest hypoxia activated NADPH oxidase via AT1 receptor and PKCα and δ. SOD protects against superoxide-mediated cytotoxicity by rapidly dismutating O^{2-} to H_2O_2. Treatment of hypoxia decreased Cu/Zn-SOD and Mn-SOD protein expression level (Fig.1). The treatment of HDL resulted in decreased ROS generation, and subsequently attenuated hypoxia-impaired superoxide dismutase (SOD) activity and suppressed ROS-induced intracellular signaling pathways (Fig.3). Hypoxia is a prevalent cellular stress in many diseased states and stimulates MAPK signaling pathways. (Bogoyevitch, Gillespie-Brown et al. 1996; Sugden and Clerk 1998; Cook, Sugden et al. 1999; Nakano, Baines et al. 2000). Our results showed that hypoxia increased p-P38 and p-ERK protein expression, but not p-JNK, and HDL can decreased the expressions.

In summary, the present results indicated that HDL attenuates hypoxia-induced cardiomyocyte apoptosis and oxidative dysfunction via modulating mitochondria dependent pathway (Fig.3, 4). Therefore, reducing the downstream of superoxide-induced ROS generation and impairment of antioxidant enzymes. In addition, HDL inhibited hypoxia-induced cell death and apoptosis in cardiomyocytes (Fig.4).

Further studies are required to confirm the effect of HDL on the inhibition of hypoxia mediated pro-atherogenic effects and the effectiveness in vivo. Our findings may provide a relevant therapeutic molecular mechanism in the improvement of cardiovascular disease. In H9c2 cells, hypoxia trigger AT1 receptor and NADPH oxidase activity. Whether HDL protects the cells against hypoxia-induced apoptosis via blocked AT1receptor or through HDL compose such as Apo A-1, SR-B1 or other receptors will be another issue we can identify in the future.

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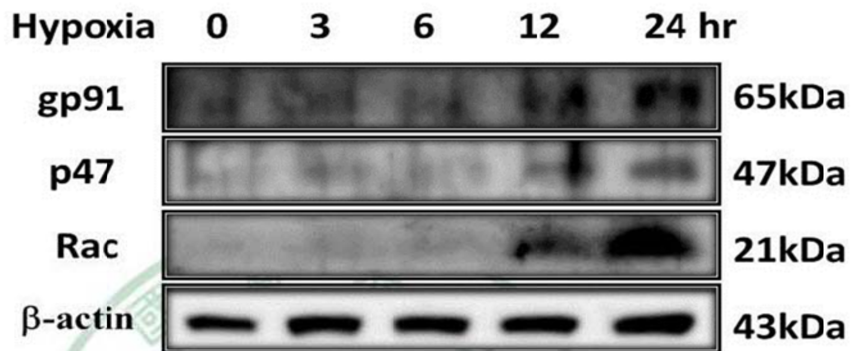
Figures

Fig.1

(A)



(B)

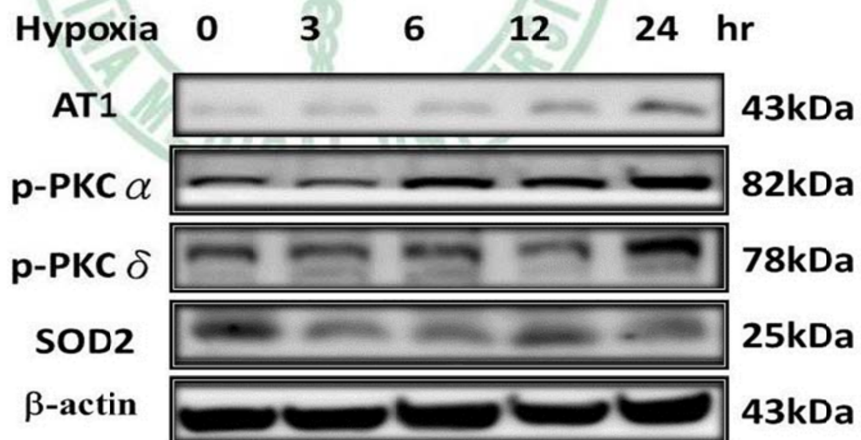


Fig.1 Hypoxia-increased oxidative stress in H9c2 cells. For Western blot analysis, H9c2 cells were treated with 1% hypoxia for 0-24h. (A) The level of NADPH oxidase (Nox2-gp91^{phox}, p47^{phox}, and Rac) protein expressions. (B) The level of AT1 receptor, p-PKCα and p-PKCδ, and antioxidant enzyme SOD2 protein expressions .

Fig.2

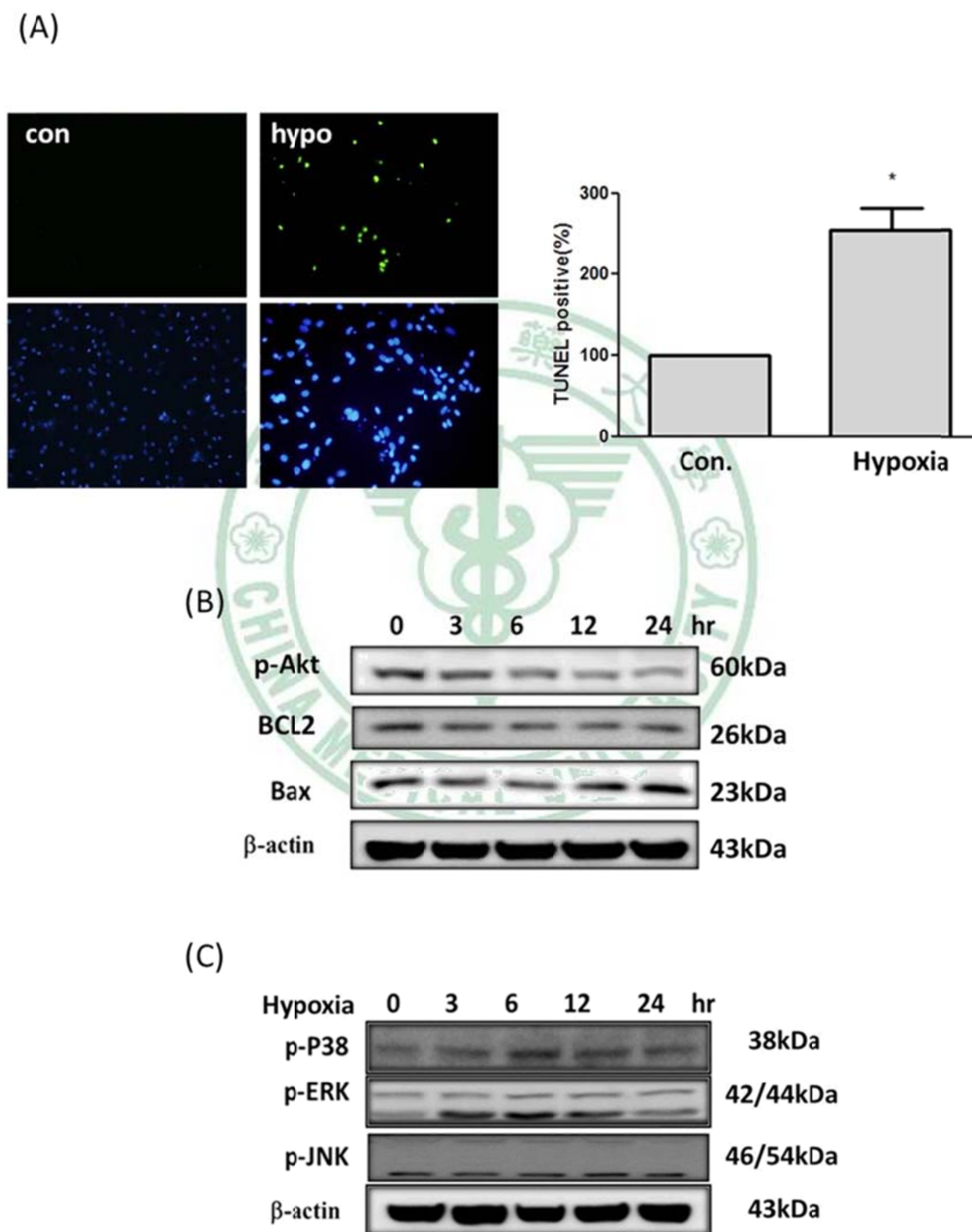
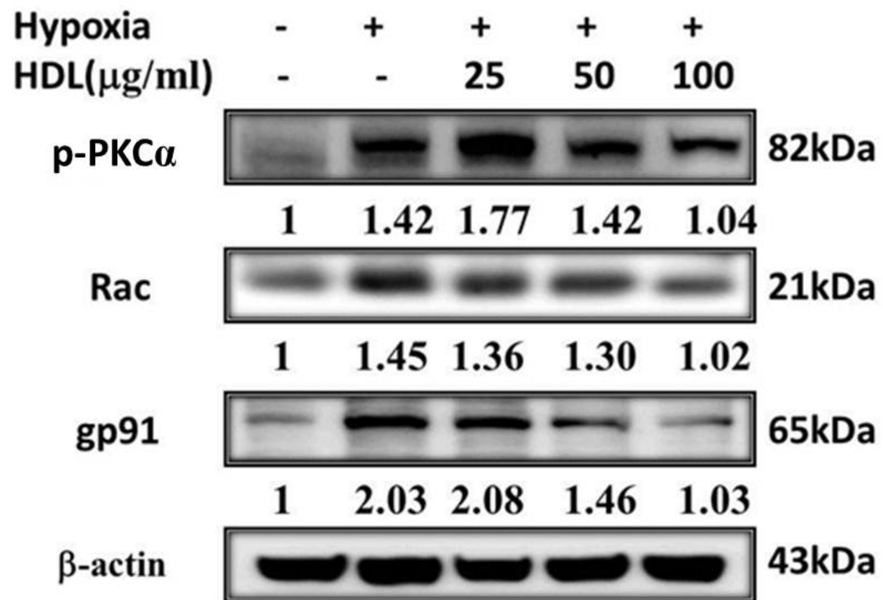


Fig.2 Effect of hypoxia-induced apoptosis and activation of MAPK family proteins, and survival proteins in H9c2 cells. (A)Fluorescence

images show the cells stained with 4,6-diamidino-2-phenylindole (DAPI) and stained using terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay. H9C2 cells were incubated with 1% hypoxia for 24 h. Data showed the means±SEM of 3 independent analyses.*P<0.05 vs. hypoxia alone treatment. H9c2 cells were treated with 1% hypoxia for various time periods (0-24h), and then were harvested and lysed. Immunoblotted with antibodies against (B) Survival proteins for 0-24h. (C) MAPK family (p-ERK, p-JNK, p-P38).

Fig.3

(A)



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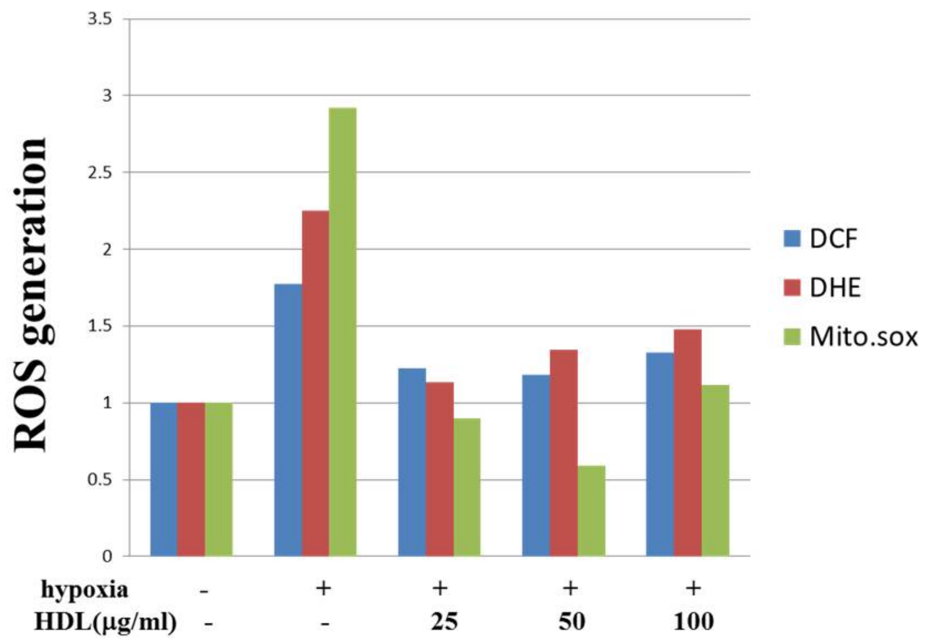


(C)

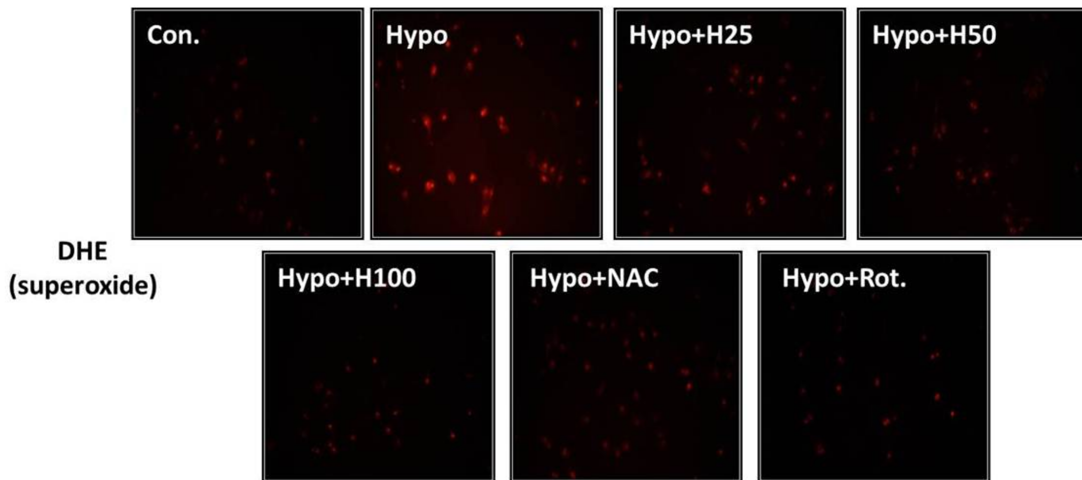


Fig.3 Inhibitory effect of HDL on hypoxia induced ROS production in H9c2 cells. H9c2 cells were treated with the indicated concentration of HDL (25-100μg/ml) for 2h, and then treated with 1% hypoxia for 24h. (A)For Western blot analysis, monoclonal anti-p-PKCα, NADPH oxidase (Nox2-gp91^{phox} and Rac), and SOD2 and anti-β-actin, antibody (for normalization) were used. (B)ROS were examined by DCF-AM (10μM), DHE (5μM), and MitoSOX™ (5μM). Fluorescence intensity of cells was measured by flow cytometry. (C) Neonatal cardiomyocytes were treated with HDL (25-100μg/ml) for 2h, or NAC (500 μM), roteone (5 μM), and then incubated with 1%hypoxia for an additional 24h, and followed by 1h incubation with DHE (10μM). Fluorescence intensity of cells was measured by phase-contrast microscopy

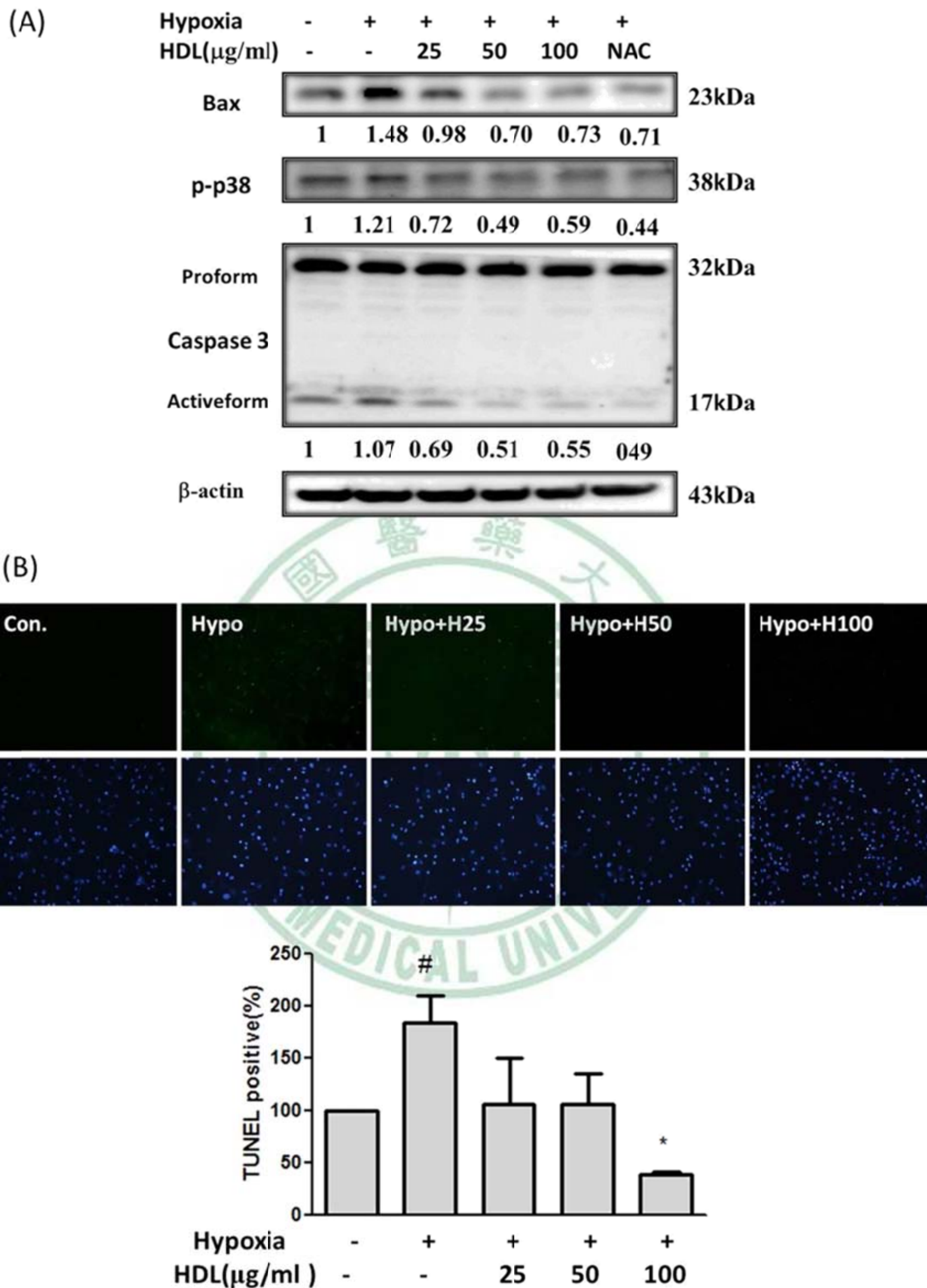
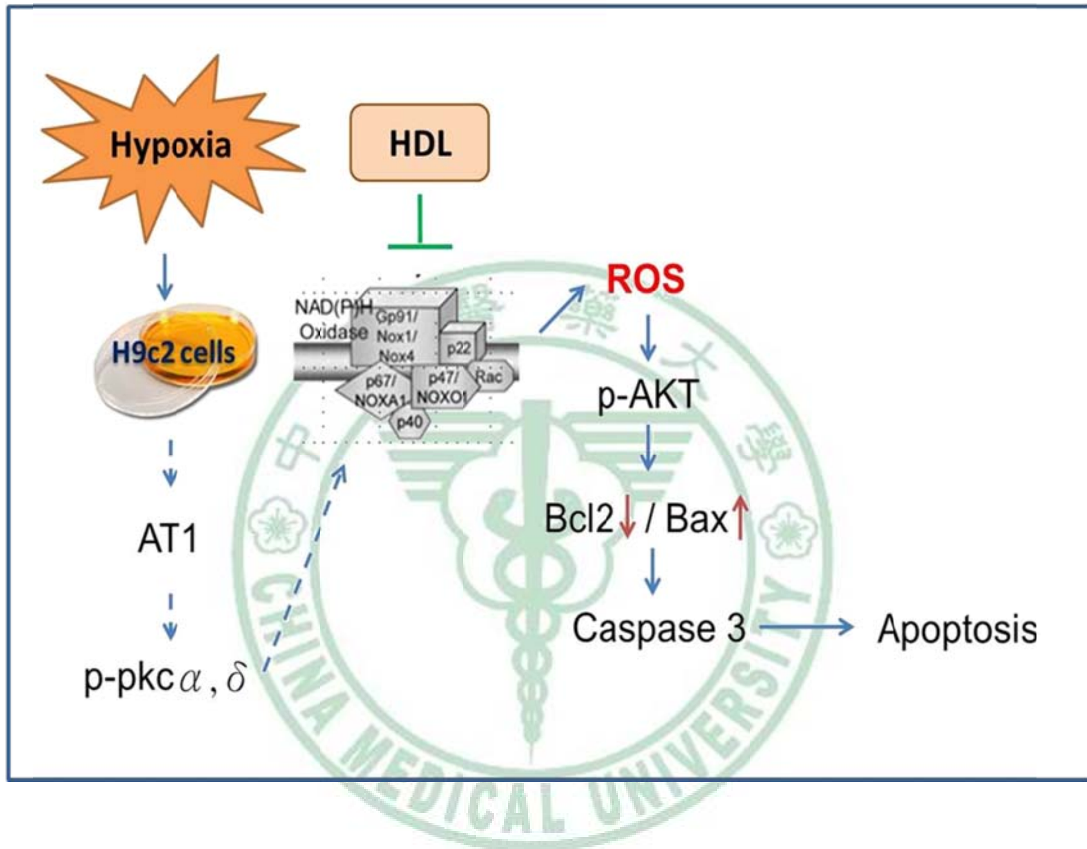


Fig.4 Inhibitory effect of HDL on hypoxia induced MAPK and apoptoic protein expressionS in H9c2 cells. H9c2 cells were treated with the indicated concentration of HDL (25-100μg/ml) for 2h, followed by

incubation of hypoxia were incubated to medium for 24h., and then were harvested and lysed. (A) For Western blot analysIs, monoclonal Bax, p-p38, casepase 3 and anti-b-actin for normalization, antibodies were used. (B) Fluorescence images shows the cells stained with 4,6-diamidino-2-phenylindole (DAPI) (upper panel) and stained using terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay (bottom panel), and photomicrographs were from phase-contrast microscopy.

Fig.5



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