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Involvement of AMPK and MAPK signaling during the progression of experimental autoimmune myocarditis in rats and its blockade using a novel antioxidant

Somasundaram Arumugam [a], Rajarajan A. Thandavarayan [a,b], Punniyakoti T. Veeraveedu [c], Vijayasree V. Giridharan [b], Vivian Soetikno [a], Meilei Harima [a], Kenji Suzuki [d], Masaki Nagata [e], Ritsuo Tagaki [e], Makoto Kodama [f], Kenichi Watanabe [a,*]

[a] Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata City 956-8603, Japan
[b] Department of Functional and Analytical Food Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata City 956-8603, Japan
[c] WPI Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan
[d] Department of Gastroenterology, Niigata University Graduate School of Medical and Dental Sciences, Niigata City 951-8510, Japan
[e] Department of Oral and Maxillofacial Surgery, Niigata University Graduate School of Medical and Dental Sciences, Gakkocho-dori 2-5274, Niigata 951-8514, Japan
[f] First Department of Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata City 951-8510, Japan

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ABSTRACT

There are various reports suggesting the role of angiotensin (Ang) receptor blockers, Ang converting enzyme inhibitors, calcium channel blockers, diuretics and antioxidants against the progression of experimental autoimmune myocarditis (EAM) to dilated cardiomyopathy (DCM). Most of them were reported to be effective during this adverse cardiac remodeling. Recently much attention has been paid to studying the involvement of AMP-activated protein kinase (AMPK) and mitogen activated protein kinase (MAPK) in various cardiovascular ailments. AMPK acts as a master sensor of cellular energy balance via maintenance of lipid and glucose metabolism. Evidences also suggest the relation between AMPK and oxidative stress during physiological and pathological myocardial cellular function. Since, it is of interest to identify the roles of AMPK and MAPK during the progression of EAM to DCM and also the effect of edaravone, a novel free radical scavenger, against its progression. For this, we have carried out western blotting, histopathological staining and immunohistochemical analyses to measure the myocardial expressions of AMPK signaling and oxidative stress related parameters in normal and vehicle or edaravone-treated EAM rats, respectively. We identified the myocardial levels of phospho Akt and phosphoinositide 3-kinase, which are the upstream proteins of AMPK and MAPK activation and both were up-regulated in the vehicle-treated rats, whereas candesartan treatment significantly reversed these changes. We have also measured the myocardial levels of p-AMPKα, different isoforms of protein kinase C and MAPK signaling proteins. All of these protein levels were significantly elevated in the hearts of DCM rats whereas edaravone treatment significantly reversed these changes. In viewing these results, we can suggest that along with MAPK, AMPK signaling also plays a crucial role in the progression of EAM and it can be effectively blocked by the treatment with a novel antioxidant, edaravone.

Introduction

Cardiovascular disorders are more prevalent all over the world as compared to other diseases and it is a fact that severe heart failure is more predominant than most cancers. Also, cardiovascular disease is one of the leading causes of death worldwide, accounting for 16.7 million deaths per annum (Rohini et al., 2010). Myocarditis is an inflammation of heart muscle and it is most often due to infection by common viruses, with an inflammatory infiltrate and damage to the heart (Baughman, 2006). It can also occur due to autoimmune reactions (Cooper, 2009). Myocarditis can be induced in rats by immunization with cardiac myosin (Kodama et al., 1990), which resembles the human giant cell myocarditis and is well characterized by extensive myocarditis necrosis, congestive heart failure and appearance of multinucleated giant cells (Kodama et al., 1994).

The heart is capable of utilizing a variety of substrates to produce the necessary ATP for cardiac function. Many cardiovascular-related disorders, such as pathological cardiac hypertrophy, heart failure, myocardial ischemia, diabetic cardiomyopathy, and lipotoxic heart disease are associated with alterations in cardiac energy metabolism (Dolinsky and Dyck, 2006). Fatty acids are the primary energy substrate of the adult heart. Switches in myocardial substrate utilization and energy production rates have been shown to occur in various cardiomyopathies, as well as in any subsequent heart failure (Taha and Lopaschuk, 2007). It has been found that the AMPK, a master sensor of cellular energy balance in mammals plays a significant role in

* Corresponding author. Fax: +81 250 25 5021.
E-mail address: watanabe@nupals.ac.jp (K. Watanabe).

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maintaining the cardiac metabolic function through activation of energy producing pathways and suppressing energy consuming processes (Sambandam and Lopaschuk, 2003). Since AMPK is central to the regulation of cardiac energy metabolism, the regulation of AMPK may be important in these various pathological settings (Dolinsky and Dyck, 2006). AMPK activation may be essential for adaptation of cardiac energy metabolism to acute and/or minor metabolic stresses, it is unknown whether AMPK activation becomes maladaptive in certain chronic disease states and/or extreme energetic stresses. However, alterations in cardiac AMPK activity are associated with a number of cardiovascular-related diseases such as pathological cardiac hypertrophy, myocardial ischemia, glycogen storage cardiomyopathy, and Wolff-Parkinson-White syndrome, suggesting the possibility of a maladaptive role. There are various reports stating that the activation of AMPK has both pro- and anti-apoptotic roles in the heart of various experimental animal models (Hickson-Bick et al., 2000; Meisse et al., 2002). Therefore, in this study we studied the possible role of AMPK in chronic heart failure induced by experimental autoimmune myocarditis (EAM).

Edaravone, a novel free radical scavenger, may be an effective agent for the attenuation of myocardial inflammation by combating oxidative stress (Tada et al., 2003). Various reports suggest the protective effect of edaravone against myocardial complications during cardiovascular disorders. Reduced myocardial infarct size and improved cardiac function and left ventricular (LV) remodeling were reported with 14 days edaravone treatment after myocardial infarction (Maruyama et al., 2006). Interestingly, edaravone treatment significantly attenuated pressure overload-induced cardiac hypertrophy mediated through its antioxidative function (Tsujimoto et al., 2005). From our lab, we have also reported the protective effects of edaravone against EAM induced by cardiac myosin, focusing on oxidative stress, endoplasmic reticulum stress, inflammatory cytokines and myocardial apoptosis (Shimazaki et al., 2010). But it is now of interest to identify whether treatment with edaravone can inhibit the progression of EAM into DCM, if so, we can study the involvement of AMPK signaling cascade and other signaling proteins during this protection.

Materials and methods

Chemicals

Unless otherwise stated, all reagents were of analytical grade and were purchased from Sigma (Tokyo, Japan). Edaravone was generously provided by Mitsubishi Research, Japan. Monoclonal antibodies of anti-rabbit phospho Akt, anti-rabbit phospho p38-mitogen activated protein kinase (MAPK), anti-rabbit phospho MAPKAPK2, anti-rabbit phospho c-Jun NH_2 kinase (JNK), anti-mouse phospho extracellular ligand regulated kinase (ERK)-1/2, anti-rabbit phosphoinositide 3-kinase (PI_3K), anti-rabbit phospho AMPKα, anti-rabbit AMPKα and anti-rabbit glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were obtained from Cell signaling. Other monoclonal antibodies used in this study include: anti-rabbit protein kinase C (PKC)-α, anti-rabbit PKC-β_1, anti-rabbit PKC-β_2 and anti-rabbit PKC-δ, which were purchased from Santa Cruz biotechnology.

Animals

Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). All experiments were carried out using 8-week-old male Lewis rats (Sukumaran et al., 2010) and were performed in accordance with the guidelines of our institute. Animals were kept in the departmental animal house under controlled conditions of 25 ± 2 °C, relative humidity of 60 ± 5% and a light–dark cycle of 12:12 h. They were fed with food pellets (Oriental Yeast Co., Ltd, Tokyo, Japan) and water ad libitum.

Immunization and treatment protocol

Lewis rats were injected in the footpads with antigen-adjuvant emulsion according to the procedure described previously (Kodama et al., 1990). In brief, porcine cardiac myosin was dissolved in phosphate-buffered saline at 5 mg/ml and emulsified with an equal volume of complete Freund's adjuvant with 11 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Lab., Detroit, MI, USA). EAM was induced in rats by immunization with 0.1 ml emulsion once by subcutaneous injection into their rear footpads (0.1 ml to each footpad). The morbidity of EAM was 100% in rats immunized by this procedure (Kodama et al., 1990; Veeraveedu et al., 2008). Four weeks after immunization, the rats were divided into three groups and received intraperitoneal injections of edaravone (3 mg/kg/day; Group Ed 3 and 10 mg/kg/day; Group Ed 10) or vehicle (Group V) for 4 weeks. Age-matched Lewis rats without immunization were used as normal controls (Group N). The doses used in the experiments were determined on the basis of the previous report suggesting their cardioprotective effects (Tsujimoto et al., 2005).

Hematoxylin-eosin (H-E) staining

After being embedded in paraffin, several transverse sections were prepared from the ventricle, and stained with H-E. Infiltration of inflammatory cells was examined in the H-E stained slides viewed under a high-power light microscope at 200× magnification. The cardiomyocyte diameter was measured from the stained slides at 400× magnification by measuring 100 cells per slide and calculating the average diameter of each cardiomyocyte using light microscopy (CIA-102, Olympus, Tokyo, Japan).

Western immunoblotting

This procedure was carried as per the method published elsewhere (Thandavarayan et al., 2009, 2010). Briefly, the myocardial tissue samples obtained from the experimental animals were homogenized with lysis buffer. Protein concentrations in these homogenized samples were measured by the bicinchoninic acid method. For western blots, proteins were separated by SDS-PAGE and identified with the following monoclonal antibodies to quantify the myocardial levels of proteins: phospho AMPKα, AMPKα, PI_3K, phospho p38 MAPK, phospho ERK1/2, phospho Akt, phospho JNK, phospho MAPKAPK2, PKC-α, PKC-β_1, PKC-β_2, PKC-δ and GAPDH. We used 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, CA, USA), and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk or 5% BSA (Sigma, St. Louis, USA) in TBS-T (20 mM/L Tris, pH 7.6, 137 mM/L NaCl, and 0.05% Tween). After incubation with the primary antibody, the bound antibody was visualized with the respective HRP-coupled secondary antibody (Santa Cruz Biotechnology) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample to check for equal loading of the sample. Films were scanned, and band densities were quantified with densitometric analysis using the Scion Image program (GT-X700, Epson, Tokyo, Japan).

Statistical analysis

All values are expressed as the means ± SEM. Statistical analysis of differences between the groups was performed by one-way ANOVA, followed by Tukey's or Bonferroni's method and the two tailed *t*-test when appropriate. $P<0.05$ was considered significant. For statistical analysis, GraphPad Prism 5 software (San Diego, CA, U.S.A) was used.