行政院國家科學委員會專題研究計畫　成果報告

Pravastatin 對於已用 CS-866 治療之心肌梗塞大鼠其心室再塑型之影響探討(2/2)

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※ （計畫名稱）

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Effect of Pravastatin on Left Ventricular Remodeling in CS866-treated Rats with Myocardial Infarction

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共同主持人：曾春典

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中文摘要:

心肌梗塞後之間隙孔重組係產生心律不整之原因。先前研究顯示 statins 可減少心律不整，其機轉未明。本研究在探討 statins 可藉由增強梗塞邊緣處之連接素 43 之表現而降低心律不整。在綁完左前降冠狀動脈之雄大鼠任意分配到 vehicle，pravastatin，mevalonate 和混和 pravastatin + mevalonate 四週，與對照組做比較，發現 pravastatin 可增強梗塞邊緣之連接素 43 之表現。共軛焦顯微鏡證實此種變化。而心律不整之分數對照組比 pravastatin 者高。但若加入 mevalonate 後，這些效果則消失。

結論：心肌梗塞後，若給予 pravastatin 可藉由增強連接素 43 之表現而降低心律不整之程度。

關鍵詞：

共軛焦顯微鏡、連接素 43、間隙孔、心肌梗塞、西方墨染

Abstract

Keywords: Confocal microscopy; Connexin43; Gap junction; Myocardial infarction; Pravastatin; Western blot.

Gap junction remodeling after infarction appears to be an important feature of anatomical substrates of ventricular arrhythmias. Statins have been shown to reduce post-operation arrhythmias. However, the involved mechanisms remain unclear. The present study was to determine whether the antiarrhythmic effect of statins is associated with an increased expression of connexin43 at the border of infarction. To elucidate the effects, we conducted experimental infarction in rats and connexin43 remodeling was investigated by immunoconfocal and Western blot analysis. After ligation of the anterior descending artery, male normocholesterolemic rats were randomized to either vehicle, pravastatin treatment, mevalonate, or combination of pravastatin and mevalonate for 4 weeks. In contrast to myocytes from the border zone in the vehicle group, which showed the amount of Cx43 proteins decreased as assessed by Western blot, pravastatin-treated rats showed significantly increased Cx43 immunostaining. Confocal microscopy confirmed the changes of the junctional complex. Arrhythmic scores during programmed stimulation were significantly higher in the vehicle than those treated with pravastatin. These beneficial effects of pravastatin were reversed by the addition of mevalonate, implicating HMG-CoA reductase as the relevant target of these

drugs.

Conclusion. The results of the present study suggest that the pravavastatin administration after infarction can reduce the inducibility of ventricular arrhythmias as a result of increased Cx43 protein expression, which is linked to mevalonate metabolismThis article provides guidance for report writing under the Grant of National Science Council beginning from fiscal year 1998.

Keywords: Research Project, Report Style, National Science Council

Introduction

Cardiac remodeling was associated with myocardial hypertrophy and reactive-reparative fibrosis and increased mortality following myocardial infarction.[1] Prolongation of action potential duration is the major cellular electrophysiological abnormality associated with left ventricular hypertrophy.[2,3] This abnormality is not uniformly distributed and is thought to be responsible for dispersion of refractoriness and increased vulnerability to ventricular arrhythmia.[4,5] Action potential prolongation in response to hypertrophy is the result of a summation of changes in the transient outward current as well as the delayed and background rectifier current.[3] Besides, left ventricular hypertrophy is accompanied by a structural remodeling of the myocardium that includes interstitial and myocardial fibrosis.[6] This structural remodeling interrupts the activation wave and trends to induce ventricular arrhythmias.[7]

Connexin43 (Cx43) is the 43-kDa member of a conserved family of membrane spanning gap junction proteins, of which Cx43 is the principal junctional protein in mammalian myocardium.[8] More than a dozen unique gap junction proteins have been cloned.[9] Each connexin subunit has four transmembrane domains in proceeding from the N- to the C-terminus, which are both localized on the cytoplasmic membrane face. Gap junctional organization is an important determinant of intracellular conductance and the conduction properties of myocardium.[10,11] Gap junction mediates cell-to-cell movement of ions, metabolites and cell signaling molecules and may play important roles in synchronized vasoactive responses, growth responses and second-messenger signaling.[12] The normal pattern of anisotropic conduction in ventricular myocardium, by which conduction parallel to the myocardium long axis is up to four times more rapid than that transverse to it is dependent in part on the low resistivity of the gap junctional membranes, their distribution, and their abundance.[13] A reduction in gap junctional coupling between myocytes may be an important morphological feature that could interact with altered membrane properties in diseased myocardium. Increases in resistivity can reduce conduction velocity and increase heterogeneity of conduction.[14] The combination of slow conduction and dispersion of action potential duration promotes reentrant tachycardia initiation and perpetuation.[15] Peters et al[16] have demonstrated that disruption of Cx43 could be a cause of arrhythmogenic nonuniformity of anisotropic conduction after infarction. At the border zone of myocardial infarcts, viable cardiomyocytes undergo reorganization of cell-cell and cell-extracellular matrix interactions as they lose cell-cell connections because of the death of neighboring cardiomyocytes and subsequently anchor to scar tissues. Impaired intercellular conduction caused by such disorganization of gap junctions can delay conduction in specific areas of the

hearts with resultant re-entry and re-excitation, which cause arrhythmias.

Epidemiological studies have demonstrated that the benefit of statin treatment after myocardial infarction extends to the patients with normocholesterolemia.[17] Although there are limited data in answering the question of when to start statin treatment after myocardial infarction, small studies[18,19] have shown that starting statin treatment as early as possible because they do indeed lead to substantially reduced mortality. Statins have been proved to inhibit the effect of Angiotensin II on cellular function including cellular hypertrophy and cardiac fibrosis.[20] Therefore, the study was aimed to elucidate the possible contribution of pravastatin to cardiac phenotypic modulation and we also explored the downstream functional significance of reduced ventricular hypertrophy by ventricular pacing in a rat myocardial infarction model. To differentiate further the direct cardiac actions of non-cholesterol from its indirect cholesterol-mediated effects, we also gave rats receiving a nonspecific (mevalonate) agonist for formation of cholesterol.

Animals.

Male normocholesterolemic Wistar rats that weighed 250-300 g fed a normal sodium diet and offered tap water ad libitum before the study. They were kept in cages, 5 per cage, in a standard light/dark room at a constant temperature (22 ± 1^0C) and humidity. On the study day (D0), 24 hours after myocardial infarction induced by ligating the left anterior descending artery, rats were randomly separated into four groups of 10 rats: (1) vehicle group; (2) pravastatin (5 mg/kg per day) in the drinking water; (3) mevalonate (50 mg/kg per day) in the ground rat chow; and (4) pravastatin + mevalonate. The drugs were used for 4 weeks starting on the day of randomization. Sham operation served as controls. The study duration was designed to be 4 weeks because the majority of the myocardial remodeling process in the rat (70-80%) is complete within 3 weeks.[21] Procedures for animal care, surgery, and euthanasia were approved by our institutional review committee for animal experiments.

Experimental myocardial infarction.

To create the model, rats were anesthetized with ketamine (90 mg/kg) intraperitoneally. After adequate anesthesia they were intubated with a 14-gauge polyethylene catheter and ventilated with room air using a small animal ventilator (model 683, Harvard Apparatus, Boston, MA). The heart was exposed via a left-sided thoracotomy, and the anterior descending artery was ligated using a 5-0 silk between the pulmonary outflow tract and the left atrium. The muscle and skin were closed in layers. Sham rats underwent the same

procedure except the suture was passed under the coronary artery and then removed.

Hemodynamics and Infarct size measurements

After the last arterial pressure measurement, the rats were anaesthetized with thiopental sodium (50 mg/kg, ip). A left thoracotomy was performed through the intercostal space. The LV apex was immediately punctured using a 25-gauge fluid-filled needle attached to a pressure transducer. Left ventricular end-systole and end-diastole pressure were measured without damped wave forms. Next, the heart was rapidly excised and suspected for retrograde perfusion with a Langendorff apparatus. At completion of the electrophysiological tests, a 1 to 1.5 mm coronal section, taken from the equator of the heart, was fixed in 10% formalin and embedded in paraffin for determination of infarct size. Each section was stained with hemotoxylin and eosin, and trichrome. The areas of scar and nonscar regions were measured the tracings by computerized planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MD) at the same mid-papillary slice of each heart. The infarct size was determined according to method of Pfeffer et al:[22] the lengths of scar for the endocardial and epicardial surfaces were summed as endocardial and epicardial circumferences. The rest of the tissue was divided in right ventricle, septum, infarcted, or adjacent noninfarcted left ventricular wall.

Perfusion of isolated hearts

Each heart was perfused with a modified Tyrode's solution containing (in mM): NaCl 117.0, $NaHCO_3$ 23.0, KCl 4.6, NaH_2PO_4 0.8, $MgCl_2$ 1.0, $CaCl_22$ 2.0, and glucose 5.5, equilibrated at 37^0C and oxygenated with a 95% O_2-5% CO_2 gas mixture. The perfusion medium and the chamber containing the isolated heart was maintained at a constant temperature of 37^0C. A peristaltic pump (LKB Bromma 12000 Varioperspex) was interposed in the aorta of the circuit that could deliver lysates at a constant flow rate (4.0 mL/min). The perfusion circuit was maintained at 37^0C by connection in series to a circulator. The aortic pressure will be 100 mm Hg and connect to a high fidelity microtip catheter (Gould Inc.) on the Gould physiological recorder. Epicardial electrograms were recorded by an atraumatic unipolar electrode, placed on the anterior left ventricular wall 2 mm below the circumflex artery.

Spontaneous and Induced arrhythmias

After isolation, the hearts were observed for 20 minutes to allow stabilization of hemodynamics. During the period, spontaneous arrhythmias were recorded. Induced arrhythmias were effected using an electrical Bloom stimulator. Stimulation intensity was twice the threshold, and

stimulus length was 5 msec. The protocol for pacing was modified from that of Nguyen et al.[23] The heart was stimulated with a train of eight stimuli at a cycle length of 100 ms (S_1), followed by one to three extrastimuli (S_2, S_3, and S_4) at shorter coupling intervals. The end point of ventricular pacing was induction of ventricular tachyarrhythmia consisting of at least 6 consecutive ventricular extrastimulus beats. An arrhythmia score was used to evaluate the incidence and duration of different arrhythmias by giving a grade to the animals as follows: 0 = no arrhythmia; 1 ≤ 10 sec ventricular tachycardia (VT), no ventricular fibrillation (VF); 2 = 11 to 30 sec VT, no VF; 3 = 31 to 90 sec VT, no VF; 4 = 91-180 sec VT, and/or no VF; 5 ≥ 180 sec VT and/or > 19 sec VF; 6 = irreversible VF.

Western Blot Analysis

Left ventricles were homogenized with a kinametic polytron blender in100 mM Tris HCl, pH 7.4, supplemented with 20 mmole/L EDTA, 1 mg/ml pepstatin A, 1 mg/ml antipain, and 1 mmole/L benzamidin. Homogenates were passed through a syringe to break down the DNA and centrifuged at 3,000*g* for 10 minutes to pellet the intact cells and elastic fibers. Supernatants were collected and centrifuged for 60 minutes at 100,000 *g* and 4^0C. That pellet was resuspected in a buffer containing 62.5 mmole/L Tris, pH 8.0, 20% SDS, and 10 mmole/L EDTA. Protein concentration was determined with the BCA protein assay reagent kit (Pierce). Twenty μg protein was separated by 8% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After incubation with rat monoclonal antibodies generated to a peptide containing amino acids 252-270 in the C-terminal of rat connexin43 (Chemicon), the nitrocellulose membrane was then rinsed with Tris-buffered saline and incubate for 1 hours at room temperature. Cx43 protein was detected using a 1:1000 dilution of Cx43 monoclonoal antibody conjugated to alkaline phosphatase in 5% non-fat milk in Tris-buffered saline. Antigen-antibody complexes were detected with 5-bromo-4-chloro-3-indolyl-phosphate and nitrblue tetrazolium chloride (Sigma). Prestained low molecular weight markers were used to identify the electrophoretic mobility of Cx43. Films were volume-integrated within the linear range of the exposure using a scanning densitometer.

Confocal microscopy

In order to investigate the spatial distribution and quantification of Cx43, analysis of confocal microscopy was performed on left ventricular muscle from adjacent and remote areas. Sectioning was performed at a thickness of 7 μM. The slides containing the sectioned tissues were rehydated in 0.01% sodium bicarbonate at pH 7.4. Sections were blocked with 0.1 mmole/L L-lysine in PBS containing 0.1% triton X-100 for 45 minutes. Tissues were incubated with mouse monoclonal anti-Cx43 antibody (Chemicon International Ltd) at dilution 1:1000 in 0.5% BSA in PBS overnight at 37^0C. The second antibody was monoclonal sheep anti-mouse biotenylated antibody/streptavidin Texas red (Amersham Life Sciences), both at 1:250

dilution in PBS containing 0.5% BSA for 1 hour. The sections were washed for 20 minutes and mounted in 50% glycerol containing para-phenrlenediamine as an antibleaching agent. Primary antibody was omitted and run in parallel in controls.

Immunolabelled sections were examined through the use of confocal laser scanning microscopy (LSM-410 Invert, Zeiss) at an excitation wavelength of 488 nm. Each transmural section was examined at a low power to determine the overall tissue architecture and amount and distribution of Cx43 label and at a higher power to detect the precise distribution at the cellular level. Each test area was digitized into a 1,024 X 1,024 matrix.

Statistical Analysis

Results were presented as mean ± SD. Densitometric analyses of Western were performed with a scanner. Differences among the groups of rats were tested by a one-way ANOVA. Subsequently analysis for significant differences between the two groups was performed with a multiple comparison test (Scheffe's method). The correlation between continuously distributed variables were tested by univariate regression analysis. Discriminant analysis was used to test the correlations between the levels of gap junction, systolic blood pressure, and the occurrence of VF. The significant level was assumed at value of $P<0.05$.

Results

The infarct size was similar among the groups, suggesting that suppression of arrhythmia was not the result of differences in infarct areas. Pravastatin do not lower serum cholesterol in rats, consistent with the notion that compensatory increases in hepatic enzyme production were observed in rats treated with statins.[26] \

Hemodynamics

Mean blood pressure and LV systolic pressure significantly lower in infarcted rats than in the sham-operated rats. Pravastatin-treated rats had significantly lower LV end-diastolic pressure.

Confocal microscopy

In sham-operated rats, gap junctions were located at the intercalated disc and no gap junctions were distributed along the lateral cell borders, consistent with prior description.[27] However, the pattern of gap junction distribution changed in infarcted hearts. Gap junctions were distributed in a relatively uniform manner along the perimeter of the myocytes. Viable cells close to and sometimes interdigitating with necrotic cells of the infarcted region showed extensive Cx43 labeling of lateral cell borders. The proportion of Cx43 label was significantly lower in infarcted rats 4 weeks after operation. Treatment with pravastatin prevented such significant decrease.

Western blot

The increase in amounts of Cx43 in pravastatin-treated rats was confirmed and quantified by Western blot, in agreement with the findings of the immunoconfocal analysis. The amount of Cx43 protein was significantly reduced to 56 ± 7% of those of sham-operated hearts ($P<0.0001$). The intensity of labeling for Cx43 was increased

39% in extracts from rats treated with pravastatin.

Electrophysiological stimulation

All sham-operated hearts contracted vigorously throughout the study and arrhythmia scores were very low. In contrast, ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in rats with MI. Pravastatin treatment decreased the inducibility of ventricular tachyarrhythmias, and there was a significant reduction in arrhythmia scores in the group compared with those in the control and combination group of pravastatin and mevalonate.

Discussions

The present study shows for the first time that long-term administration of pravastatin reduced susceptibility of pacing-induced arrhythmias after myocardial infarction. The results of Western blot analysis (total Cx43 protein amount) and confocal microscopy (abnormal distribution of gap junction) indicated a change in gap junction area and distribution, which could account for the antiarrhythmic effect after statin administration. The favorable effect of pravastatin was completely prevented in the presence of mevalonate, which indicates that the effect was specific to the 3-hydroxy-3-methyglutaryl-CoA reductase pathway. These data supports the critical role of the gap junction channel in maintaining cardiac electrical stability.

Pravastatin and Cx43

The mechanisms by which pravastatin modulates gap junction protein remain to be defined. Clearly, pravastatin did not exert any hemodynamic effects at the dose used in this study. No differences in heart rates among the groups, suggesting that attenuation of Cx43 expression has not significant effect on sinus node. The finding was consistent with the notion that Cx43 is not expressed in the nodes of the cardiac conduction system.[28]

The changes in gap junction after MI associated with an increased propensity for ventricular arrhythmias strongly suggest their role in arrhythmogenesis. Gap junction remodeling has been observed in a variety of heart diseases, including infarction.[29] Gap junction remodeling is a potential mechanism leading to ventricular arrhythmias and sudden death. Mechanistically, regional downregulation of Cx43 after infarction is theorized to cause a loss of synchronized ventricular conduction and ultimately arrhythmias. The density of Cx43 expression has shown to be crucial in coordinated conduction in the border zone where malignant arrhythmia origins.[29] This study is an extension of the work of Luke and Saffitz,[30] showing that reduced distribution of intercellular connections in the healed infarct border zone. The decrease in Cx43 expression of 56 ± 7% in the present study is consistent with previous studies, showing that Cx43 protein content is reduced by 40-70% at the border zone.[31,32] The significant reduction of Cx43 delineated with confocal microscopy in border zone tissues indicates that theses myocytes are relatively uncoupled and that the tissue would have increased passive intercellular resistance and enhance the anisotropy of intercellular connections. Furthermore, gap junctions were diffusely distributed along the side of myocytes after infarction assessed by confocal microscopy. The structural inhomogeneity might trigger arrhythmias by enhancing the generation of early afterdepolarization.[33] The residual Cx43 coupling may allow for the propagation of early afterdepolarization. Taken together, changes in the distribution and density of gap junctions per se after infarction provide

substrates to develop ventricular tachycardia induced by reentry or triggered activity.

Our results were consistent with previous studies, showing that statins upregulated gap junction functions.[34] Remodeling of Cx43 after MI is a complex process involving perturbations of Cx gene expression and Cx protein synthesis and degradation. The signaling pathways to trigger remained unclear, but likely involve alteration in levels of free radical and angiotensin II, both of which were elevated during the process of ventricular remodeling. Free radicals have been identified as a factor that inhibits Cx43 expression in rat hepatocytes.[35] Antioxidants prevented the inhibition of gap junction communication between hepatocytes.[36] Wagner et al[37] have shown that statins can inhibit endothelial free radicals by preventing the isoprenylation of p21 Rac, which inhibits Rac1 translocation from the cytosol to the membrane and, thus blocks the assembly of membrane bound NADPH oxidase. Statins, which inhibit function of the small G proteins, may attenuate generation of free radicals by blocking signaling pathways. Second, angiotensin II upregulates gap junctions in cultured neonatal rat ventricular myocytes by increasing Cx43 synthesis.[38] On this basis, it might corresponding be predicted that pravastatin might reduce Cx43 expression as an effective blocker of angiotensin II.[39] However, it was not the case. Inhibition of pravastatin-induced angiotensin II did not play a significant role in modulation of Cx43 expression. Taken together, the antioxidant effect of pravastatin played an important role in enhanced expression of Cx43 protein after **MI.**

Conclusions

This study provided a novel target in the treatment of patients at risk for lethal ventricular arrhythmias.

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