行政院國家科學委員會專題研究計畫　期中精簡報告

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(1/2)

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行政院國家科學委員會專題研究計畫期中進度報告

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(1/2)

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

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中文摘要：

關鍵字：心律不整，肥厚，心肌梗塞，心肌細胞

心肌梗塞後之心肌細胞增厚係引起心律不整之重要原因。血管收縮素接受體拮抗劑和 statins 二者皆可降低心血管之死亡率。二者併用時，是否會比單一使用在減少心肌肥厚效果上更好呢？不得而知。

在綁完左前降冠狀動脈後，大鼠分成餵食 CS866 (0.01, 0.1, 1 和 2 mg/kg per day)或 pravastatin (5 mg/kg per day)或兩者共計四週。此兩種藥當單獨給予時皆可降低梗塞邊緣的心肌肥厚現象。若兩者合用時，則減低效果更明顯。

心肌之內皮素-1 在邊緣區比正常組高 6.5 倍。而加入 pravastatin 後，內皮素濃度則下降。當併用低濃度的 CS866 (0.01 mg/kg per day) 比單獨使用 pravastatin 更能減低心肌細胞肥厚變化。而使用最高濃度的 CS866 (2 mg/kg per day)併用者，則有最明顯的減低心肌肥厚變化，雖然其內皮素-1 濃度沒有明顯地下降。而心律不整之分數反映出心肌肥厚程度。

併用 pravastatin 和 CS866 可依劑量變化而減低心肌肥厚程度。此乃因透過不同之作用機轉，進而降低心律不整之發生。這種現象提供併用藥物之治療契機。

英文摘要：

Keywords: Arrhythmias; Hypertrophy; Myocardial infarction; Myocytes.

***Background*—**Reactive cardiomyocyte hypertrophy after myocardial infarction is an important risk factor for arrhythmias. Both angiotensin receptor antagonists

and 3-hydroxy-3-methylgultaryl coenzyme A (HMG-CoA) reductase inhibitors have been shown to decrease cardiovascular morbidity and mortality. Whether combination treatment may be superior to either drug alone on cardiomyocyte hypertrophy remains unclear.

***Methods and Results*—**After ligation of the left anterior descending artery, rats were randomized to both, 1, or neither of the angiotensin receptor antagonist Cs866 (0.01, 0.1, 1, and 2 mg/kg per day) and HMG-CoA reductase inhibitor pravastatin (5 mg/kg/day) for four weeks. Each drug, when given alone, decreased cardiomyocyte sizes isolated by enzymatic dissociation at the border zone compared with vehicles. However, compared with either drug alone, combined Cs866 and pravastatin prevent cardiomyocyte hypertrophy to a larger extent. The myocardial endothelin-1 levels at the border zone were 6.5-fold higher (P <0.0001) in the vehicle group compared with the sham group, which can be inhibited after pravastatin administration. Further evaluation of combination therapy with a low dose of Cs866 (0.01 mg/kg/day) significantly prevented cardiomyocyte hypertrophy compared with pravastatin alone (3020 ± 368 vs. 3202 ± 406 μm^2 in the pravastatin-treated group, P = 0.04). With the highest dose of Cs866 (2 mg/kg/day) in combined therapy, we observed a further reduction of cardiomyocyte hypertrophy although tissue endothelin-1 levels remained stable in combination groups. Measurements of arrhythmic score mirrored those of cardiomyocyte hypertrophy.

***Conclusions*—**Dual-therapy with pravastatin and Cs866, which produced an additive reduction in cardiomyocyte hypertrophy in a dose-dependent manner after myocardial infarction through different mechanisms, decreases the propensity of the heart to arrhythmogenesis. Because cotreatment with statins and Cs866 acts in an additive manner, these observations provide important therapeutic implications in pharmacotherapy of clinical practice.

Introduction

Cardiac remodeling was associated with myocardial hypertrophy and left ventricular (LV) dilation following myocardial infarction.[1] These changes in LV geometry contribute to the development of depressed cardiac performance, arrhythmias and sudden cardiac death.[1] Accumulating evidence indicates that angiotensin II plays a key role in the pathophysiology of myocardial hypertrophy after myocardial infarction.[2] Angiotensin receptor blockers (ARBs) favorably modulate extracellular signal-regulated protein kinase to elicit attenuated cardiac hypertrophy.[3] There is considerable evidence that electrophysiological changes were associated with the hypertrophied myocardium.[4] Hypertrophied myocardium has been shown to generate arrhythmias more readily than normal tissue.[4] Agents with the regression of ventricular hypertrophy have been shown to decrease the susceptibility of ventricular arrhythmias.[5] Recent trials attributed the survival benefit of ARB to reduction of arrhythmic death in animals[6,7] and in patients.[8]

We have previously demonstrated that pravastatin can attenuate ventricular hypertrophy separate from their cholesterol-lowering actions in hyperlipidemic patients.[9] 3-hydroxy-3-methylgultaryl coenzyme A reductase inhibitor (statin) therapy has many effects independent of changes in plasma cholesterol concentrations, such as attenuated endothelin-1 (ET-1) expression.[10] An alteration in the expression of ET-1 has been proposed as a potential regulation of cell hypertrophy after infarction, given their crucial role in a key mediator to activate *Ras* proteins as a mitogen.[11] It has been reported that statins can inhibit ET-1 and reduced ET-1 concentrations effectively prevents cellular hypertrophy.[12] Although hypertrophy is reduced by administering ARBs, it is not arrested, and thus it may not sufficiently antagonize disease progression and thereby reduce the risk of major cardiac events.[13] Thus, it is of interest to identify drugs other than ARBs to reduce cardiac hypertrophy. In fact, angiotensin II and ET-1 have been shown to have a mutual reciprocal signaling pathways to trigger ventricular hypertrophy. As yet, however, the usefulness of combination therapy with these 2 agents after infarction has not been demonstrated, and the pharmacological mechanisms underlying the benefit of the combination therapy are not defined. Thus, we assessed whether pravastatin provides an additive effect on ventricular hypertrophy by inhibiting ET-1 expression after myocardial infarction in animals cotreated with Cs866, a nonpeptide ARB. Because regional performance reflects morphological differences, we explored the downstream functional significance of reduced ventricular hypertrophy by ventricular pacing in a rat model of myocardial infarction.

Methods (實驗方法)

Animals

Male normocholesterolemic Wistar rats that weighed 300-350 g fed a normal sodium diet, and offered tap water ad libitum. 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, 24 hours after myocardial infarction induced by ligating the left anterior descending artery, rats were randomly assigned into 7 groups: (1) vehicle group; (2) pravastatin (5 mg/kg per day, Sankyo Co., Tokyo, Japan) in the drinking water; (3) Cs866 (2 mg/kg per day, Sankyo Co., Tokyo, Japan) given orally by gastric gavage once a day; and (4) pravastatin (5 mg/kg per day) + Cs866 (0.01, 0.l, 1, and 2 mg/kg per day). The dose of Cs866 more than 0.1 mg/kg per day has been used to inhibit AT_1-receptor-induced pressor response.[14] To differentiate the hemodynamic and angiotensin effect of Cs866 on ventricular remodeling, a non-pressor dose (0.01 mg/kg per day) was administered. The drugs were started 24 hours after myocardial infarction, during which drugs can exert maximum benefits at this timing window.[15] In each-treated group, drugs were withdrawn about 24 hours before the end of the experiments in order to eliminate their pharmacological actions. 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.[16] Sham operation served as controls.

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

Functional parameters were measured in anesthetized rats at the end of the study. Using a 2F micromanometer-tipped catheter (Model SPR-407, Miller Instruments, Houston, TX) inserted through the right carotid artery, we measured LV systolic and diastolic pressure as the mean of measurements of five consecutive pressure cycles. 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:[17] the lengths of scar for the endocardial and epicardial surfaces were summed as endocardial and epicardial circumferences. It has been shown that infarct size is larger than 30% of the LV, hypertrophy of the residual myocardium progresses after infarction.[17] Thus, with respect to clinical importance, only rats with infarction larger than 30% of the LV were selected for analysis.

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, $CaCl_2$ 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 was maintained at a constant temperature of 37^0C with a constant flow at 4 ml/min. Epicardial electrograms were recorded by an atraumatic unipolar electrode, placed on the epicardial surface of the right ventricle and anterior LV wall 2 mm below the ligation site of the anterior descending artery. The electrocardiograms were continuously displayed on a Gould recorder at 5 mm/sec chart speed and a HP oscilloscope (Hewlett Packard, 54503A) at 100 mm/sec sweep speed.

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 delivered at the necrotic area and at the normal right ventricle. The protocol for pacing was modified from that of Nguyen et al.[18] Induction of ventricular arrhythmias was then attempted by ventricular stimulation at a basic cycle length of 150 ms (S_0) with single (S_1), double (S_2), and triple (S_3) extrastimuli delivered after 8 paced beats. The end point of ventricular pacing was induction of ventricular tachyarrhythmia consisting of at least 8 consecutive ventricular extrastimulus beats. A preparation was considered non-inducible when pacing produced either no ventricular premature contraction or only self-terminating salvos of <6 beats. Ventricular tachyarrhythmias including ventricular tachycardia and ventricular fibrillation were considered nonsustained when it lasted ≤15 beats and sustained when it lasted >15 beats. An arrhythmia scoring system was used as previously described.[18]

Immunohistochemical analysis of ET-1

In additional to endothelial cells, various cells including cardiomyocytes have the ability to synthesize ET-1.[19] In order to investigate the spatial distribution of ET-1, immunohistochemical staining was performed on LV muscle at the border zone (0 to 2 mm outside the infarct) and non-ischemic areas (>2 mm outside the infarct). Hearts were snap-frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek), and cryosections were performed at a thickness of 5μm. The slides containing the sectioned tissues were fixed in 10% formalin and rinsed them in PBS. Immersed the sections in 3% H_2O_2 for 12 minutes at room temperature for blocking endogenous peroxidase activity. Sections were blocked with 10% normal goat serum in PBS for 15 minutes. Tissues were incubated with a rabbit polyclonal anti-ET-1 antibody (Immuno-Biological Lab Co., LTD., Gunma, Japan) at dilution 1:10 in 1% normal goat serum in PBS overnight at 4^0C. Immunostaining with ET-1 antibodies was performed using a standard immunoperoxidase technique (N-Histofine Simple Stain Rat MAX PO kit, Nichirei Co., Tokyo, Japan). The antibody used had been tested for specificity in the rat. Negative controls were performed by omitting the primary antibody. Because of the wide variability of structural composition of border zone regions which resulted in intercellular connection ranging from total disruption in fully scarred regions to negligible alterations with normal appearing myocytes, we selected samples for analysis that were composed of cardiomyocytes separated by diffuse interstitial fibrosis.

Plasma and tissue levels of ET-1 and plasma cholesterol levels

Because of a local release of ET-1, blood samples from the aortic root and the tissue at the border zone and remote interventricular zone were obtained for measurements of systemic and local ET-1 levels at the end of the study. Plasma ET-1 concentration was measured by collecting 4 ml of blood in test tubes containing 2% ethylenediaminetetraacetic acid (80 μl/ml of blood). Blood samples were immediately centrifuges at 3,000*g* for 10 minutes, and the plasmas were stored at -70^0c until further analysis. The myocardiums were homogenized with a polytron homogenizer for 60 seconds in 10 vol. Of 1 mol/l acetic acid containing 10 μg/ml pepstatin and immediately boiled for 10 minutes at 4^0C. ET-1 was measured by immunoassay (R&D System Inc, Minneapolis, MN). Plasma (1 ml) was acidified with 3 ml of 4% acetic acid and ET-1 was extracted with a Sep-pak C-18 cartridge. The detection limit was 1 pg/ml for ET-1. Cross-reactivity with ET-2, ET-3 and big ET-1 was 0.01%, 7.8%, and 0.02%, respectively. Intra-assay and inter-assay coefficients of variation was 4.5% and 6.6%, respectively. Cholesterol was measured in plasma by an automated method.

Cell isolation

Since the infarct size measurement procedure does not permit quantitation of cardiomyocyte sizes, additional groups of rats were infarcted using the same procedures and used for measurement of cell sizes at the end of the study. Myocytes were enzymatically isolated according to previously described techniques.[20] Briefly, the rats were heparinized; and heart excised and perfused at a constant flow of 8 ml/min by a modified Langendorff technique at 37^0C with a nominally Ca^{2+}-free, oxygenated Tyrode solution (pH 7.4) containing (in m*M*): NaCl 137, KCl 5.4, $MgCl_2$ 1.1, dextrose 11, HEPES 10. After 5 min of equilibration, the perfusion was changed to the same solution containing 0.34 mg/ml collagenase (type II; Sigma Chemical Co., St, Louis, Mo., USA) and 0.08 mg/ml protease (type XIV, Sigma). After 10 to 15 min of digestion, the residual enzyme-containing solution was cleaned by 5-min perfusion with 0.2 m*M* Ca^{2+} Tyrode solution. Then, the heart was removed from the cannula, the undigested infarct area was removed, and the border zone was mechanically dispersed. Random high-power fields of the rod-like relaxed myocytes with clear striations were selected in phase contrast illumination mode of confocal microscopy (LSM-410 Invert, Zeiss) to eliminate selection bias. At least 10 cells from each section were selected for measurement of cell length, width and area, and the mean value was used as the individual value for each section. Although it is impossible to isolate myocytes from hearts subjected to confocal study, infarct size should be considered to be similar within various groups because animals were randomly assigned to cell size or confocal study. In the sham-operated group, cell width and length were measured from the ventricular free wall for comparisons.

Statistical Analysis

Results were presented as mean ± SD. A two-way ANOVA was used to search for possible effects of pravastatin and Cs866 on the measurements of hemodynamics, ET-1 levels, cholesterol levels, myocyte sizes and, if an F value was found to be significant, a two-tailed Student's *t*-test for paired observation with Bonferroni's correction was used to test differences. The interaction term of pravastatin and Cs866 effects was incorporated into the model. Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal-Wallis test followed by a Mann-Whitney test. Correlation between the degree of attenuated cardiomyocyte hypertrophy and the dose of Cs866 in the combined therapy was assessed by Pearson's correlation coefficient. The significant level was assumed at value of $P<0.05$.

Results (結果)

Differences in mortality between vehicle and treated groups were not found

throughout the study. 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.[21] These data indicate the nonlipid effect of pravastatin on ventricular remodeling.

Hemodynamics

Although the infarct size was not affected by either drug administration, their treatment ameliorated the elevated end-diastolic pressure. Compared with the vehicle group (108 ± 10 mm Hg), LV systolic pressure was significantly reduced by Cs866 at 2 mg/kg (87 ± 6 mm Hg, P=0.002), and by combined Cs866 at 0.1, 1, and 2 mg/kg and pravastatin at 5 mg/kg (99 ± 8, 90 ± 10, 90 ± 6 mm Hg, respectively, all P<0.05). However, there was no significant difference in systolic blood pressure in the groups treated with pravastatin alone (105 ± 8 mm Hg), and combined Cs866 at 0.01 mg/kg and pravastatin (106 ± 12 mm Hg) compared with the vehicle group. Heart rate did not differ among the groups.

Morphometric studies

Body weights were unchanged by infarction or treatment. Compared with sham-operated rats in the vehicle group, there was a significant increase in right ventricular weight/body weight ratio, and lung weight/ body weight ratio. Four weeks after infarction, the infarcted area of the LV was very thin and was totally replaced by fully differentiated scar tissue. The weight of the LV inclusive of the septum remained similar 4 weeks after coronary artery occlusion among the infarcted groups.

To characterize the cardiac hypertrophy on a cellular level, we isolated cardiomyocytes from different treated groups. After infarction, cell area increased by 65% (P<0.0001) at the border zone compared with sham-operated group. Pravastatin (5 mg/kg per day, 32%) or Cs866 (2 mg/kg per day, 29%) alone reduced cell areas compared with the vehicle group. However, compared with each agent alone, their combination decreased cell sizes to a greater extent. Compared with the group treated with pravastatin alone (3202 ± 406 μm^2), the magnitude of attenuated cardiomyocyte hypertrophy was significantly increased in combination therapy even at the non-pressor dose of Cs866 (0.01 mg/kg, 3020 ± 368 μm^2, P=0.04), suggesting that Cs866 provides additionally beneficial effect on attenuated cardiomyocyte hypertrophy independent of hemodynamic changes. The maximal preventive effect of combined therapy of Cs866 was observed at dose of 2 mg/kg per day. The Pearson linear regression models showed that there was a trend between the reduction of cardiomyocyte hypertrophy and the dose of Cs866 (0, 0.01, 0.1, 1 and 2 mg/kg) in the combined therapy (reduction in cardiomyocyte hypertrophy (%) = 3.57*the dose of Cs866 + 34.78, r = 0.82, P=0.06).

Electrophysiological stimulation

To further elucidate the physiological effect of attenuated cardiomyocyte hypertrophy, ventricular pacing was performed. Arrhythmia scores in sham-operated rats were very low (0). In contrast, ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in infarcted rats. Either pravastatin or Cs866 treatment decreased the inducibility of ventricular tachyarrhythmias. Compared with monotherapy, their combination more potently suppressed the inducibility of ventricular tachyarrhythmias.

Circulating and myocardial ET-1 levels

Circulating ET-1 levels remained similar in infarcted rats among the groups. To investigate the possible role of cardiac ET-1 synthesis, we determined the ventricular ET-1 levels. Expression was region dependent with a significant increase at the border zone (7.85 ± 3.11 pg/mg protein) compared with that in the interventricular septum (2.43 ± 1.12 pg/mg protein, $P<0.0001$) in the vehicle group. ET-1 levels at the border zone were significantly lower in the pravastatin-treated rats than in the vehicle-treated rats (2.72 ± 0.83 vs. 7.85 ± 3.11 pg/mg protein, $P<0.0001$). The suppressive effect of the combination of Cs866 (0.01, 0.1, 1, and 2 mg/kg) and pravastatin was similar to that of pravastatin alone in terms of tissue ET-1 levels at the border zone.

Immunohistochemical analyses

Immunohistochemical analysis of the infarcted myocardium revealed the presence of ET-1 immunoreactivity in the myocardial tissue. In the vehicle group, a marked increase in the intensity of ET-1 immunostaining was observed at the border zone compared with remote interventricular regions, consistent with the results of tissue ET-1 levels.

The interstitial cells and the endothelial and smooth muscle cells of the intramyocardial coronary arteries showed modest staining for ET-1, and the intensity did not differ between the vehicle group and the sham group. Thus, the elevated ET-1 in the infarcted hearts may be attributable to ET-1 synthesis in cardiomyocytes. The number of cardiomyocytes showing positive immunoreaction to ET-1 was low and the intensity of the immunoreaction was reduced in pravastatin-treated groups compared with that in the vehicle group.

Discussions (討論)

To our knowledge, the combined effect of Cs866 and pravastatin on attenuated cardiomyocyte hypertrophy has not been reported earlier. Our present work provided solid experimental evidence that combination therapy with ARB and pravastatin is more beneficial than each agent alone for attenuated cardiomyocyte

hypertrophy in a dose-dependent manner and reduced arrhythmic score. This additive effect of Cs866 and pravastatin suggests that both agents improve ventricular remodeling by fundamentally different mechanisms.

Our conclusions are supported by 3 lines of evidence: 1) Either ARBs or statins can similarly attenuate cardiomyocyte hypertrophy. 2) The synthesis of ET-1 in cardiomyocyte was reduced when they were exposed to pravastatin, both in the absence and presence of Cs866, suggesting an ET-1-independent effect of Cs866 and ET-1-dependent effect of pravastatin on cardiomyocyte remodeling. The capacity to inhibit angiotensin II activity is not the only factor responsible for the antihypertrophic effect in this model and pravastatin has additional mechanisms of action at the myocardial level to inhibit ET-1 overexpression. The finding further supports the notion that combination therapy of pravastatin and Cs866 is synergic in their effect on attenuated cardiomyocyte hypertrophy after infarction through different mechanisms. 3) With the highest dose of Cs866 (2 mg/kg/day) in combined therapy, we observed a further reduction of cardiomyocyte hypertrophy compared with lower dose of Cs866, implying a dose-dependent effect of Cs866 in combination therapy.

Conclusions

Our present work provided the first evidence that both ARBs and statins use different actions to exert a similar effect on attenuation of cardiac hypertrophy in a manner independent of their antihypertensive and hypolipidemic effects. Because cardiac hypertrophy is hard to normalize by either ARBs or statins alone, the present study proposes a good indication for combination therapy with ARBs and statins against cardiac remodeling after myocardial infarction. Characterization of the cardioprotective mechanisms provides a rationale for the future design of combination drugs in the second prevention of coronary artery disease.

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