行政院國家科學委員會補助專題研究計畫成果報告

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行政院國家科學委員會專題研究計畫成果報告

Effects of Angiotensin-Converting Enzyme Inhibitor/Angiotensin II blocker on Arrhythmias and Expression of Cardiomyocyte Gap Junction in Post-Myocardial Infarction Rats

血管收縮素轉換酶抑制劑和血管收縮抑制劑在心肌梗塞後之大鼠對心律不整和心肌間隙孔之影響

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一、中文摘要

先前研究顯示血管收縮素轉換酶抑制劑可明顯地降低心肌梗塞後之死亡率。此種死亡率之降低有部分來自於心律不整之下降。甫刻定雖無直接的電氣生理作用，但卻可使不正常之電氣現象回歸正常。血管收縮素轉換酶抑制劑和血管收縮抑制劑有類似的臨床效果，但血管收縮抑制劑卻比前者對心肌梗塞倖存者有較好的存活率。

間隙孔是心臟細胞間重要的傳導組織。可以藉此調控細胞間離子交換，代謝產物和訊號傳遞。因此對於生長調節和胞內訊號傳遞非常重要。Connexin43（Cx43）是哺乳動物心臟中最重要的間隙孔組成份。間隙孔減少會造成形態上的改變，並使細胞間傳導阻力增加，減緩傳導速度。如此的改變會造成迴路頻脈。本研究之目的在探討(一) 在長期給予血管收縮素轉換酶抑制劑和血管收縮抑制劑對間隙孔之影響。(二) 並探討這二種抑制劑對間隙孔之差別。(三) 在不同劑量的血管收縮素轉換酶抑制劑是否對間隙孔之表現有不同差異。

雄大鼠在綁完前降冠狀動脈後，隨意分列下列五組：(1) 對照組，(2) 低劑量甫刻定(5mg/kg/day)，(3) 中劑量甫刻定(25mg/kg/day)，(4) 高劑量甫刻定(50mg/kg/day)，(5) Losartan (10mg/kg/day)。

結果顯示心律不整分數以對照組比餵刻甫定或 losartan 者高，此種變化恰反應出 Cx43 之變化。結果：本研究顯示心肌梗塞後餵食刻甫定或 Losartan 皆可使心律不整降低，其因係增加 Cx43 之故。

關鍵詞：甫刻定；共軛顯微鏡；間隙孔；心臟內超音波；心肌梗塞；北方墨染；西方墨染

二、計畫成果摘要：

Abstract

Recent trials attributed the survival benefit of ACE inhibitors to reduction of arrhythmic death. Captopril induced the normalization of electrical abnormalities although captopril does not appear to have direct cardiac electrocardiophysiological effects. Although the similar effects between ACE inhibitor and angiotensin II blockers were noted in clinical use, an unexpected survival benefit confined to a reduction in sudden cardiac death was observed in the losartan group compared with the captopril group. However, the involved mechanism remains unclear. Gap junction in mammalian heart function to provide low-resistance channels between adjacent cells for passage of ions and small molecules. Increases in resistivity can reduce conduction velocity and increase heterogeneity of conduction. The combination of slow conduction and dispersion of action potential duration promotes reentrant tachycardia initiation and perpetuation. Therefore, the study was aimed to assess whether the expression of Cx43, the physiologically predominant connexin of myocardium, is

altered during the chronic administration of ACE inhibitor or angiotensin II blocker. To explore the survival differences between ACE inhibitor and angiotensin II blocker in patients with myocardial infarction, we compare the density of Cx43 after chronic administration. In addition, we examined whether ACE inhibitor in vivo, independent of blood pressure, contributes to cardiac connexin43 modulation with different dosages of ACE inhibitor.

After ligation of left anterior descending artery, rats were randomly separated into five groups of 30 rats (10 rats per timed point): (1) vehicle group; (2) low-dose captopril (5 mg/kg per day); (3) intermediate-dose (25 mg/kg per day); (4) high-dose captopril (50 mg/kg per day); and (5) Losartan (10 mg/kg per day). Sham operation will serve as controls. Four weeks after randomization, rats were sacrificed after obtaining basic hemodynamics. The isolated hearts underwent spontaneous and induced arrhythmia analysis.

Arrhythmic scores during programmed stimulation were significantly higher in the vehicle group than those treated with either low or high doses of captopril or losartan. Rats treated with captopril had significant increased amount of Cx43 compared with those from the vehicle group. In fact, the amount of Cx43 assessed by Western blot was inversely parallel with arrhythmic scores

Conclusion. The results of the present study suggest that either captopril or losartan administration after infarction can reduce the inducibility of ventricular arrhythmias as a result of increased Cx43 protein expression in a dose-independent manner.

Keywords: Captopril; Confocal microscopy; Connexin43; Gap junction; Intracardiac ultrasound; Losartan; Myocardial infarction; Northern blot; Western blot.

三、計畫簡介（Introduction）

Introduction

After myocardial infarction, ventricular remodeling occurs which may be an adaptive process to the loss of myocytes, and consists of hypertrophy of the remaining cardiomyocytes (1). This reactive hypertrophy assists in maintaining the cardiac output. However, pathologic remodeling occurs in which dilation and resultant disorganization of intercellular coupling combine to initiate deterioration in arrhythmias. Angiotensin II plays a key role in this process (2). Angiotensin converting enzyme (ACE) inhibitors significantly reduce mortality in survivors of myocardial infarction (3,4). Early trials attributed the survival benefit of ACE inhibitors predominantly to a retardation of hemodynamic deterioration (3). Recent trials attributed the survival benefit of ACE inhibitors to reduction of arrhythmic death in animals (5) and in patients (6). Captopril induced the normalization of electrical abnormalities although captopril does not appear to have direct cardiac electrocardiophysiological effects (7,8). Although several possible mechanisms for antiarrhythmic effects of ACE inhibitor have been proposed (9), the antiarrhythmic effects of captopril could be due to indirect actions through intercellular communications. Previous studies have suggested that ACE inhibitors are able to modulate intercellular resistance through ACE-independent actions although the involved mechanisms still remain unclear (10,11). Studies have demonstrated that enalapril increased junctional conductance of cardiac myocytes by approximately onefold within 2 minutes (11). Angiotensin II reduces the junctional conductance by about 55% within 30 seconds. Although the similar effects between ACE inhibitor and angiotensin II blockers were noted in clinical use, conversion of angiotensin I to angiotensin II continues only in patients administered with ACE inhibitor because this reaction also can be catalyzed by chymase in the heart. Whether the difference explains an unexpected survival benefit confined to a reduction in sudden cardiac death in the losartan group compared with the captopril group remained unclear (13).

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 (15). More than a dozen unique gap junction proteins have been cloned (16). 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 (17,18). 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 (19). 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 (20) is dependent in part on the low resistivity of the gap junctional membranes, their distribution, and their abundance. 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 (21). The combination of slow conduction and dispersion of action potential duration promotes reentrant tachycardia initiation and perpetuation (22). Peters et al (23) have demonstrated that disruption of cx43 could be a cause of arrhythmogenic nonuniformity of anisotropic conduction after infarction. The present study was designed to compare the effects of ACE inhibitor and angiotensin II blockers on gap junction in a rat model of remodeled myocardial infarction.

四、材料及方法(Subjects and Methods)

Animals.

Procedures for animal care, surgery, and euthanasia were approved by our institutional review committee for animal experiments. Male normotensive Wistar rats that weighed 250-300 g fed a normal sodium diet, with a sodium content of 0.32 wt% 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 $\pm$ 1^{0}C) and humidity. On the study day (D0), after ligation of the left anterior descending artery, rats were randomly separated into 4 groups of 60 rats (15 rats per group): (1) vehicle group; (2) captopril (5 mg/kg per day); (3) Losartan (5 mg/kg per day); (4) EXP 3174 (5 mg/kg per day). All drugs were dissolved in the drinking water. Another 4 groups of sham operation with the same treatment serve as controls. The drugs were used for 4 weeks starting on the day of randomization. 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 (24).

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 will be ligated using a 7-0 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

Hemodynamic parameters were measured in lightly anesthetized rats via the abdominal aorta at the end of the study. After the arterial pressure measurement, a left thoracotomy was performed through the intercostal space. The LV apex was immediately punctured using a 24-gauge fluid-filled catheter attached to a pressure transducer. LV end-systole and end-diastole pressure was measured without damped wave forms. Next, blood samples were obtained from the abdominal aorta for cholesterol measurement and the heart was rapidly excised and suspected for retrograde

perfusion with a Langendorff apparatus. At completion of the electrophysiological tests, the atria and the right ventricles were trimmed off, and the LV was rinsed in cold physiological saline, weighed, and immediately frozen in liquid nitrogen after obtaining a coronal section of the LV for infarct size estimation. A section, taken from the equator of the LV, 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 (24): the lengths of scar for the endocardial and epicardial surfaces were summed as endocardial and epicardial circumferences. With respect to clinical importance, only rats with large MI (>30%) were be 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°C and oxygenated with a 95% O_2-5% CO_2 gas mixture. The perfusion medium was maintained at a constant temperature of 37°C 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 atrium and anterior LV wall 2 mm below the circumflex artery. A bipolar pacing electrode was placed near the apex of the heart on the anterior epicardial surface of the right ventricle. Atrial and ventricular epicardial electrocardiograms were continuously displayed on a Gould recorder at 5 or 250 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 contraction and rhythm. During the period, electrocardiograms were recorded for QRS duration measurement by averaging 5 QRS intervals. The protocol for pacing was modified from that of Belichard et al (25). Stimulation intensity was twice the threshold, and stimulus length was 5 msec. Programmed right ventricular stimulation was performed by delivering a train of 8 beats at a basic cycle length of 150 ms followed by delivery of an extrastimulus (S_2) by 10 ms decrements. The longest interval at which S_2 fails to evoke a depolarization is termed the effective refractory period. The end point of ventricular pacing was induction of ventricular tachyarrhythmia. A preparation was considered non-inducible when pacing produced either no VPC or only self-terminating salvos of < 6 beats. Ventricular tachyarrhythmias including ventricular tachycardia (VT) and ventricular fibrillation (VF) were considered nonsustained when it lasted ≤ 15 beats and sustained when it lasted > 15 beats. In the rat, distinction between VT and VF is difficult because both arrhythmias can convert into each other and VF can terminate spontaneously. An arrhythmia scoring system was used as previously described (25). The experimental protocols were typically completed within 10 minutes.

Western Blot Analysis of Cx43

Samples of the left ventricle from the border zone and interventricular (non-ischemic) areas were cut transmurally to include all layers from the epi- to the endocardium, were frozen rapidly in liquid nitrogen, and stored at –80°C until use. Western blot analysis was performed as previously described (26). Each lane was loaded with 20 μg of total protein. Immunoreactivity on blots was detected by 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride. Films were volume-integrated within the linear range of the exposure using a scanning densitometer. Experiments were replicated three times.

Statstical Analysis

Results were presented as mean ± SD. Densitometric analyses of Western and Northern blots 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 was 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 $P<0.05$.

The occurrence of MI was associated with a reduced body weight gain, which was similar in the infarcted rat groups. The infarct size was similar among the groups, suggesting that suppression of arrhythmia was not the result of differences in infarct areas.

Hemodynamics

Mean blood pressure and LV systolic pressure significantly lower in infarcted rats than in the sham-operated rats. Captopril and losartan-treated rats had significantly lower LV end-diastolic pressure compared with that in the control group.

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 either captopril or losartan prevented such significant decrease.

Western blot

The increase in amounts of Cx43 in captopril-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 increased to 125% of those of sham-operated hearts ($P<0.05$).

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. Captopril or losartan 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. The effective refractory period was similar among the groups.

五、結果與討論

The present study shows for the first time that long-term administration of captopril or losartan reduced susceptibility of pacing-induced arrhythmias after myocardial infarction by increased amount of Cx43 expression. 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 captopril or losartan administration. These data supports the critical role of the gap junction channel in maintaining cardiac electrical stability.

Captopril, losartan and Cx43

The mechanisms by which captopril or losartan modulates gap junction protein remain to be defined. 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).

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 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 (31). The residual Cx43 coupling may allow for the propagation of early afterdepolarization. Besides, the interfaces between myocytes with remodeled gap junctions were the location where functional lines of unidirectional block and reentry formed. Although the safety factor for conduction has been shown to paradoxically increase with reduced gap junction coupling (32), it may also facilitate reentry. 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.

Remodeling of Cx43 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 (33). Antioxidants prevented the inhibition of gap junction communication between hepatocytes (34). Previous studies have shown that captopril can act as free radical scavengers (35). Second, angiotensin II upregulates gap junctions in cultured neonatal rat ventricular myocytes by increasing Cx43 synthesis (36). On this basis, it might corresponding be predicted that captopril might reduce Cx43 expression as an effective blocker of angiotensin II. However, it was not the case. Inhibition of captopril-induced angiotensin II did not play a significant role in modulation of Cx43 expression. Taken together, the antioxidant effect of captopril played an important role in enhanced expression of Cx43 protein after MI.

六、Conclusions

Either captopril or losartan has been shown to be effective in the prevention of pacing-induced arrhythmias after myocardial infarction through increased expression of Cx43. This study may provide a novel target in the treatment of patients at risk for lethal ventricular arrhythmias.

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