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 on Expression of Cardiomyocyte Gap Junction and Arrhythmias in Two-kidney, One-clip Hypertensive Rats

血管收縮轉換酶抑制劑在 2K, 1C 高血壓大鼠對心肌間隙孔和心律不整之影響

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共同主持人：蔡長和　執行機構及單位名稱：台大醫院外科部
計畫參與人員：xxxxxx　執行機構及單位名稱

一、中文摘要

間隙孔是心臟細胞間重要的傳導組織。Connexin43（Cx43）是哺乳動物心臟中最重要的間隙孔組成份。長期高血壓常併發心臟肥厚。而心肌肥厚造成心肌傳導速度之改變。先前研究顯示在 2K1C 高血壓大鼠中 Cx43 有上升現象，但在人體心肌肥厚反而出現 Cx43 下降現象。臨床上每種高血壓藥劑皆有降壓效果，但對於降低心肌重量之效果則不同。如此血液動力學(血壓下降)和結構性(指心肌重量)之不平行變化，代表著某種非血液動力學之因素在心肌肥厚中扮演著重要角色。因此本實驗在探討著給予血管收縮轉換酶抑制劑是否會影響 Cx43 之表現。此種之變化是否與血壓之變化有關。

在做完 2K1C 高血壓模型後，雄大鼠隨意分配到四組：（1）不給藥，（2）低劑量 Captopril（25mg/kg），（3）正常劑量 Captopril(每天 50mg/kg)和(4)Hydralazine（每天 10mg/kg）。沒綁腎血管之雄大鼠當作對照組。

結果顯示此種腎動脈高壓引起血壓上升，造成左心室/身體重上升。雖然 Hydralazine 和 Captopril 有相似的降壓效果，但兩者對左心室/身體重之影響則不同。而心律不整分數在 Hydralazine 和對照組明顯高於 Captopril 組，並且 Cx43 之量在 Captopril 明顯較其他組增加。本實驗顯示血管收縮素在腎動脈高壓引起的左心室肥厚扮演重要角色。而 Captopril 比 Hydralazine 在抑制左心室肥厚與心律不整有效，此種效果與 Cx43 之量有關。

關鍵詞：2K1C 高血壓，共軛顯微鏡，間隙孔，心臟內超音波，北方墨染，西方墨染

二、計畫成果摘要:

Abstract

Gap junction in mammalian heart function to provide low-resistance channels between adjacent cells for passage of ions and small molecules. Connexin43 (Cx43) is the principal junctional protein in mammalian myocardium. Myocardial hypertrophy induced by hypertension is associated with alterations of myocardial conduction velocity. Previous studies showed that aortic Cx43 levels were increased during the early phase of two-kidney/one-clip hypertension in rats. In human hypertrophied myocardium, a reduction of Cx43 density has been reported. Practically all antihypertensive agents reduce blood pressure, emphasizing the importance of hemodynamic overload in the pathogenesis of hypertensive target organ damage. However, numerous short-term experimental and clinical trials have shown that various antihypertensive drugs, although achieving similar degrees of pressure reduction, differed in their ability to reduced left ventricular mass. This hemodynamic structural dissociation has suggested strongly that nonhemodynamic factors participate in the development and reversal of left ventricular hypertrophy. 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. In addition, We examined

whether ACE inhibitor in vivo, independent of blood pressure, contributes to cardiac connexin43 modulation.

After 2-kidney, 1-clip operation, male Wistar rats will be randomly separated into four groups of 20 rats for 8 weeks: (1) vehicle group, (2) low-dose captopril (25 mg/kg per day), (3) regular-dose captopril (50 mg/kg per day), and (4) hydralazine (10 mg/kg per day). Renovascular constriction increased the arterial pressure, resulting in increased left ventricle/body weight ratio. Hydralazine and captopril reduced mean blood pressure comparably yet had a differential effect on the ration of left ventricular weight to body weight. Arrhythmic scores during programmed stimulation were significantly higher in vehicle and hydralazine-treated groups than those treated with captopril. Rats in the vehicle group showed significant left ventricular hypertrophy. Rats treated with captopril had significant increased amount of Cx43 compared with those from hydralazine and control groups. In conclusion, the results suggest that cardiac renin-angiotensin system may play an important role in renovascular hypertension-induced left ventricular hypertrophy. Captopril is more effective than hydralazine in regressing left ventricular hypertrophy and reducing pacing-induced arrhythmias through increased amount of Cx43 in 2K, 1C hypertensive rats.

Keywords: 2-K,1-C hypertension; Captopril; Confocal microscopy; Connexin43; Gap junction; Hydralazine; Intracardiac ultrasound; Northern blot; Pacing; Western blot.

三、計畫簡介（Introduction）

Hypertension results in left ventricular hypertrophy which is associated with an increased risk of ventricular arrhythmia. Prolongation of action potential duration is the major cellular electrophysiological abnormality associated with left ventricular hypertrophy.[1,2] This abnormality is not uniformly distributed and is thought to be responsible for dispersion of refractoriness and increased vulnerability to ventricular arrhythmia.[3,4] 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.[2] Besides, left ventricular hypertrophy is accompanied by a structural remodeling of the myocardium that includes interstitial and myocardial fibrosis.[5] This structural remodeling for hypertension will interrupt the activation wave and trend to induce ventricular arrhythmias.[6]

Angiotensin converting enzyme (ACE) inhibitors provides antiarrhythmic effects in animals[7] and in patients[8]. Captopril induced the normalization of electrical abnormalities although captopril does not appear to have direct cardiac electrocardiophysiological effects.[9,10] Although several possible mechanism for antiarrhythmic effects of ACE inhibitor have been proposed,[11] 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.[12,13] Studies have demonstrated that enalapril increased junctional conductance of cardiac myocytes by approximately onefold within 2 minutes.[13] Angiotensin II reduces the junctional conductance by about 55% within 30 seconds. De Mello et al[13] showed that effects of angiotensin II on junctional conductance were blunted by exposure of the cells to enalapril prior to the addition of angiotensin II. The antiarrhythmic effects of ACE inhibitors seems to be independent of circulating angiotensin II. In fact, low doses of ACE inhibitors with little or no effect on arterial blood pressure are also effective.[14,15] However, the involved mechanisms remain unclear.

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. [16] More than a dozen unique gap junction proteins have been cloned.[17] 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.[18,19] 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.[20] 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[21] 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.[22] The combination of slow conduction and dispersion of action potential duration promotes reentrant tachycardia initiation and perpetuation.[23]

Practically all antihypertensive agents reduce blood pressure, emphasizing the importance of hemodynamic overload in the pathogenesis of hypertensive target organ damage.[24] However, numerous short-term experimental and clinical trials have shown that various antihypertensive drugs, although achieving similar degrees of pressure reduction, differed in their ability to reduced left ventricular mass.[25,26] This hemodynamic structural dissociation has suggested strongly that nonhemodynamic factors participate in the development and reversal of left ventricular hypertrophy.[27,28] Therefore, the study will be aimed to assess whether the expression of Cx43, the physiologically predominant connexin of myocardium, is altered during the chronic administration of ACE inhibitor. In addition, we examined whether ACE inhibitor in vivo, independent of blood pressure, contributes to cardiac connexin43 modulation.

四、材料及方法(Subjects and Methods)

Methodology

Procedures for animal care, surgery, and euthanasia were approved by our institutional review committee for animal experiments. Normotensive Wistar rats that weighed 150-160 g (around 7 weeks old) 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 ± 1°C) and humidity. On the study day (D0), after the operation and the transesophageal echocardioghic examination, rats were randomly separated into five groups of 20 rats: (1) Sham operation, (2) vehicle group, (3) captopril (25 mg/kg per day) in the drinking water, (4) captopril (50 mg/kg per day) in the drinking water. The dose of captopril was based on previous findings suggesting that it sufficiently inhibited *in vivo* angiotensin-converting enzyme,[29] (5) Hydralazine (10 mg/kg per day), dissolved in the drinking water, which completely normalized blood pressure.[30] The drugs were used for 8 weeks starting on the day of randomization.

Two-kidney one-clip

Sixty male rats were anaesthetized by intraperitoneal injection of thiopental sodium (Nesdonal, Specia, Rhone-Poulenc Rorer) at 50 mg/kg and silver clips, 0.25 mm internal diameter, were slipped around each renal artery as close as possible to its exit from the aorta. Sham-operated rats (n = 20) were exposed to the same surgical manipulations, except the clipping. The wound was sutured and the animals were allowed to recover. After return to their cage, all rats were maintained on a regular diet with free access to water for 8 weeks.

Blood pressure measurements

Functional parameters were measured in anesthetized rats. After 56 days, the rats were anaesthetized with thiopental sodium (50 mg/kg, ip) prior to performing hemodynamic and echocardiographic measurements. Finally, hearts were excised and perfused.

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.[31] 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.[31] The experimental protocols were typically completed within 10 minutes.

Western Blot Analysis of Cx43

Samples of the left ventricle 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.[32] 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. Signals of specific transcripts were related to the corresponding GAPDH signals and expressed relative to the lowest control ratio, which was assigned the arbitrary values of 1. 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. The significant level was assumed at value of $P<0.05$.

五、結果(Results)

LV tissue hypertrophy following 2K, 1C was estimated in 30 rats (6 rats in each group) sacrificed 8 weeks after the surgery. The heart: bogy weight and LV:body weight ratio were significantly higher in 2K, 1C rats compared with control groups.

Hemodynamics

Mean blood pressure and LV systolic pressure significantly higher in renovascular hypertensive rats than in the sham-operated rats. Either captopril or hydralazine-treated rats had significantly lower blood pressure at a similar magnitude.

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 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 hydralazine.

六、討論(Discussion)

The present study shows for the first time that long-term administration of captopril reduced susceptibility of pacing-induced arrhythmias in renovascular hypertension. 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 administration. These data supports the critical role of the gap junction channel in maintaining cardiac electrical stability.

The mechanisms by which captopril modulates gap junction protein remain to be defined. Clearly, blood pressure is not the only factor playing in the increased expression of Cx43 because hydralazine showed similar decrease of blood pressure without associated with Cx43 changes.

Gap junction remodeling has been observed in a variety of heart diseases, including infarction.[33] 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.[33] Gap junctions were diffusely distributed along the side of myocytes assessed by confocal microscopy. The structural inhomogeneity might trigger arrhythmias by enhancing the generation of early afterdepolarization.[34] 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,[35] it may also facilitate reentry. Taken together, changes in the distribution and density of gap junctions per se 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.[36] Antioxidants prevented the inhibition of gap junction communication between hepatocytes.[37] Previous studies have shown that captopril can act as free radical scavengers.[38] Second, angiotensin II upregulates gap junctions in cultured neonatal rat ventricular myocytes by increasing Cx43 synthesis.[39] On this basis, it might corresponding be predicted that captopril might reduce Cx43 expression as an effective blocker of angiotensin II.[40] 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.

七、Conclusions

Captopril has been shown to be effective in the treatment of hypertension and pacing-induced arrhythmias 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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