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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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※ **Cilostazol 對狗心肌梗塞之影響：Connexin43 之角色** ※

※ **Effects of Cilostazol, a new Phosphodiesterase Inhibitor, on Infarct Size in the Canine Heart: Role of connexin43** ※

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計畫主持人：周迺寬

共同主持人：李聰明

計畫參與人員：

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Cilostazol 對狗心肌梗塞之影響：Connexin43 之角色

Effects of Cilostazol, a new Phosphodiesterase Inhibitor, on Infarct Size in the Canine Heart: Role of connexin43

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一、中文摘要

Cilostazol 係一種治療腳血管阻塞的藥物。本身可增加血中 cAMP 之濃度，因此可能造成間隙孔連接素 43 之改變。本研究在探討 Cilostazol 是否對心臟具保護效果，而此效果是否與連接素 43 之改變有關。在綁住冠狀動脈前 15 分鐘，分別給 OSMD 或 Cilostazol。發現二組之心肌梗塞面積差不多。在心肌缺氧/梗塞時會誘發連接素 43 之去磷酸化，而此種變化二組差不多。因此我們結論：Cilostazol 並不會增加心肌梗塞面積。

關鍵詞：普達，間隙孔，收縮帶狀死亡，狗，再灌流傷害、西方墨點

Abstract

Cilostazol, a novel phosphodiesterase inhibitor, may increase intracellular cAMP of cardiomyocytes by blocking its hydrolysis, which can modulate expression of connexin43. The study purpose was to evaluate whether cilostazol provides the cardioprotection and to assess whether the cardioprotection is associated with an attenuated expression of connexin43 at the border of infarction in a canine model of acute myocardial infarction. Vehicle (n = 7) or cilostazol (n = 10, 1.8 mg/kg/min) were given intravenously 15 minutes before the coronary artery occlusion. Among survivors, infarct size was not significantly different between control and supplemented groups. Connexin43 underwent dephosphorylation in response to ischemia/reperfusion measured by Western blot in control hearts at the border zone; these changes were not significantly different by cilostazol administration. Confocal microscopy confirmed the changes of junctional complexes. It is concluded that cilostazol, at therapeutic concentrations, did not increase infarct size. This provides perspective for analysis of the pathophysiology and treatment of acute myocardial infarction.

Keywords: Adenosine; Cilostazol; Connexin43; Contraction band necrosis; Dogs; Reperfusion injury; Western blot.tyle,

二、緣由與目的

Cilostazol is a novel phosphodiesterase inhibitor that has been used for treatment of peripheral vascular disease, approved by US Food and Drug Administration. In addition to its antiplatelet and vasodilator properties in platelets and vascular smooth cells, cilostazol may also increase intracellular cAMP of cardiomyocytes by blocking its hydrolysis [1]. Patients with peripheral vascular disease are at a high risk of disability from coronary artery disease [2]. Most of patients with peripheral vascular disease have a high incidence of cardiac mortality. Because drug withdrawal worsened the walking of claudicants who have benefited from cilostazol therapy, long-term use of cilostazol was recommended [3].

Thus, the effects of cilostazol on myocardial infarction which is a potential complication in patients with peripheral vascular disease treated with such a drug should be a clinical relevance and important issue.

Previous studies have demonstrated that cAMP induces connexin43 (Cx43) cellular redistribution and clustering at membrane apposition, which may increase cell-to-cell communication [4]. 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 [5]. Gap junction mediates cell-to-cell movement of Ca^{2+} ions, which will induce calcium overloading and increase contraction band necrosis during reperfusion [6]. Cx43 is a phosphoprotein [5]. Changes in phosphorylation can affect channel function and properties [7]. Increased dephosphorylated Cx43 contributed to electrical uncoupling at the gap junction during acute myocardial ischemia [7]. Ischemia-reperfusion is thought to increase intracellular calcium concentrations by either increasing inward flux of calcium or inhibiting intracellular calcium sequestration [8]. The increased cytoplasmic calcium concentration has been shown to form contraction band necrosis and extent the infarct size [9]. Pretreatment with a calcium channel blocker had a protective effect [10], suggesting that increased intracellular calcium concentration was the cause of this structural injury. Cytosolic calcium concentrations are increased through several mechanisms, including stimulation of plasma membrane, and sarcoplasmic reticulum Ca^{2+} pump [11]. The sarcoplasmic reticulum is critical to regulation of intracellular Ca^{2+} stores and release of calcium from the sarcoplasmic reticulum is mediated by inositol (1,4,5)-triphosphate (IP_3). Previous studies have demonstrated that traffic of potentially harmful cytosolic messengers such as IP_3 between ischemic cells and surrounding nonischemic cells might cause increased injury [12], leading to myofibrillar hypercontracture and further precipitating cell death [13]. Gap junction uncouplers have been reported to exert a beneficial effect in ischemia/reperfusion models both in the myocardium [14,15] and in the brain [16]. Histological analysis revealed a more fragmented appearance of the areas of necrosis and less formation of contraction band necrosis [15]. Thus, it is logical to speculate that cilostazol which increases intracellular cAMP could extend infarct size by increased junctional permeability.

Recently, Liu et al [17] reported that cilostazol inhibits adenosine uptake into the cells, which may lead to increases in interstitial adenosine levels in the heart. Potentiation of adenosine release is effective in limiting infarct size during reperfusion after sustained myocardial ischemia. Thus, cilostazol may have benefits by an adenosine-dependent pathway. However, it remained unknown whether cilostazol will have benefits in ischemia-reperfusion conditions. No consistent cardioprotection effects among several phosphodiesterase inhibitors were observed. Pentoxifylline did not have effects on infarct size [18]. Vesnarinone showed significant limitation of infarct size in a canine ischemia-reperfusion model [19]. Thus, it is important to distinguish the differences in pharmacological effects between cilostazol and other phosphodiesterase inhibitors. The net effect of cilostazol on infarct size is difficult to predict from available studies because one would be expected to increase infarct size (increased intracellular cAMP

and enhanced Cx43 protein expression), whereas the other would be expected to decrease infarct size (increased adenosine levels). The aims of the study were (1) to assess the effects of cilostazol on infarct size; and (2) to assess whether the cardiac effect of cilostazol was related to either adenosine levels or changes of Cx43 protein expression assessed by 2 isoform-specific antibodies.

三、Methods

Preparation.

All experiments were conducted on mongrel dogs, weighing 10-15 kg. The experimental procedures have been previously described [20]. In brief, the dogs were intubated and mechanically ventilated (Bennett MA-2) by using room air delivered at a rate of 20-25 strokes per minutes and a tidal volume of 300 ml. Because epicardial temperature is a major determinant of myocardial infarct size [21], body temperature was maintained at 38 ± 0.5°C by means of a temperature probe thermometer inserted into the pericardial cradle so that its sensor surface was adjacent to the posterior surface of the heart. The thermometer was attached to a homeothermic blanket control unit (Gaymar Medi-Therm II). A standard limb-lead electrocardiogram was recorded. Left ventricular cavity and systemic blood pressures were measured with catheter-tip micromanometers inserted via the left ventricular apex and right femoral artery, respectively. The reference pressure level that was set at the right atrial level was used for all pressure measurements. Hydration was maintained by a slow 2.5% dextrose infusion. Because operation-related catecholamine release has been implicated as a mechanism of ischemic preconditioning [22], each dog had the same stabilization time (15 minutes) before the study. Then hemodynamic parameters, including heart rate, arterial blood pressure, and left ventricular end-systolic/end-diastolic pressure were continuously recorded on a 4-channel polygraph recorder (Hewlett-Packward model M1962A). IP_3 release is necessary for the propagation of intercellular Ca^{2+} waves through the gap junction communication in cardiac myocytes [23]. To prevent the confounding effect of heparin (IP_3 blocker) on Ca^{2+} wave propagation, no heparin was used throughout the study.

The chest was opened through the left fourth intercostal space, the pericardium was opened and the heart will be exposed. Near the base of the heart, the left anterior descending artery proximal to the first diagonal branch was encircled with a 4-0 silk suture. Myocardial ischemia was confirmed by regional cyanosis, acute epicardial electrocardiographic ST segment elevation and coronary flow changes detected by intravascular Doppler flowire. Reperfusion was effected by releasing the snare and was confirmed by visible hyperemia over the surface and hyperemic Doppler shift.

Experimental protocol.

The dogs were assigned to either control or cilostazol treatment. All animals were subjected to a 60-minute coronary occlusion followed by 120 minutes of reperfusion. Cilostazol at the dose of 1.8 mg/kg/min was administered 15 minutes before the onset of myocardial occlusion. The dose was chosen because the serum levels correspond with those observed clinically after oral administration of the drug to humans (100 mg b.i.d.) [24].

At the end of the protocol, the coronary artery was clamped, and 4 ml of a solution of methylene blue dye (Sigma Chemical Co., St. Louis, MO.) was injected into the left ventricular apex, defining the ischemic areas

at risk (AAR). The heart was sliced transversely into six to eight sections, and the slices were photographed to record the ischemic areas (uncolored by the blue dye) and the nonischemic, normal areas (perfused blue) in each slice. After a 10-minute incubation in a 1% solution of buffered triphenyltetrazolium chloride (TTC), the slices were again photographed to record the necrotic regions (unstained by TTC) and the noninfarcted regions (stained red by TTC). Later the photographic slides were projected and traced. The areas of ischemic and normally perfused regions and the areas of necrotic and nonnecrotic regions were measured on the tracings by computerized planimetry (Image Pro Plus, CA). Three areas were multiplied by the weight of each slice, and the results were summed to obtain the area of risk and infarction.

Histology analysis.

Extensive histological samples were taken from each transverse section, processed by conventional methods, and stained with hematoxylin and eosin, and Masson for contraction band. A pathologist who was unaware of the treatment protocol examined the samples for microscopic evidence of contraction band necrosis on random fields at a magnification of 400X. In each case the results from the section with the highest number of bands were used. The histologic severity of contraction band necrosis was used to grade injury on a scale of 0 to 3 for the number of contraction bands: 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) which represented 1, 1 to 5, 6 to 10, and 11 or more cells with contraction bands per sq mm of tissue, respectively. Reproducibility of the method for grading contraction band necrosis was assessed by analyzing interobserver and intraobserver variabilities. Interobserver variability was determined by having a second observer reanalyze staining from the original preparations analyzed by the first observer.

Western Blot Analysis

The left ventricle was rapidly infused with 50 mL diethyl pyrocarbonate (Sigma)/PBS to wash blood cells. Samples from adjacent and remote areas of the left ventricle 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 9,000*g* for 30 minutes to pellet the intact cells and elastic fibers. Supernatants were collected and centrifuged for 60 minutes at 100,000*g* and 4^0C. Supernatant 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, the nitrocellulose membrane was then rinsed with a blocking solution and incubated for 2 hours at room temperature. Cx43 protein was detected using a 1:1000 dilution of Cx43 monoclonal antibody (Chemicon) according to the manufacture's protocol. A horseradish peroxidase-linked secondary anti-mouse antibody (Amersham) was used at a final dilution of 1:1000, and the film was exposed for 1 hour. Autoradiographic bands of 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°C. 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.

Exclusion criteria.

Animals were omitted from analysis: (1) if such severe hypotension was observed that the experiment could not be continued successfully for the duration of the protocol; (2) if intractable ventricular fibrillation occurs or antiarrhythmic agents are needed to correct arrhythmia; or (3) presence of heart worms. Dogs with ventricular fibrillation during reperfusion will be resuscitated and converted to a stable rhythm by internal electric shocks (3x10 W). The low energy did not result in more cell necrosis [25]. Survival percentage was calculated as Number of Surviving Dogs /(Number of Assigned Dogs - Number of Dogs with collateral > 20%) x100.

Statistics.

Differences were tested for hemodynamic variables, infarct size and AAR among the 7 groups by nonparametric Kruskal-Wallis statistic analysis followed by a Newman-Keuls post hoc test. Densitometric quantification of signal intensity was performed for Western analysis. Background measurements of signal intensity were subtracted individually from each lane. A value of $p < 0.05$ was considered to be significant.

四、Results

Concentrations of arterial blood gas, calcium, sodium and potassium were fairly stable throughout the study.

Dog survival

A total of 19 animals were enrolled in the study. Two animals in the control group were excluded for intractable ventricular fibrillation during coronary occlusion. The remaining dogs were assigned to either control (n = 7) or cilostazol (n = 10).

Hemodynamics

Preocclusion heart rate, arterial pressure, and left ventricular end-diastolic pressure were similar between the 2 groups. After coronary occlusion, left ventricular end-diastolic pressure markedly increased, whereas heart rate and arterial blood pressure did not change significantly. The pressure rate index, an index of myocardial oxygen demand, was comparable for the 2 groups before coronary artery ligation and throughout the study.

Infarct size and AAR

There were no differences in body weight or heart weight between the 2 groups. There was no significant difference in AAR expressed as a percentage of left ventricle between the 2 groups, indicating that a comparable degree of ischemic risk. After 1 hour of coronary artery occlusion followed by 2 hours of reperfusion, the necrotic area, expressed as a percentage of the AAR, was 37 ± 8% in the control and 43 ± 10% (P = NS).

Histology analysis

Macroscopically, either control or cilostazol-treated dogs exhibited confluent infarctions. Histological analysis revealed infarcts composed almost exclusively of contraction band necrosis. The severity of contraction band necrosis was not significant differences between the 2 groups (2.6 ± 0.5 in controls *vs* 2.7 ± 0.7, P = NS).

Cx43 Western Analysis

Two predominant forms of Cx43 were detected: one nonphosphorylated form (Cx43-NP; 41 kDa) and the other phosphorylated species (Cx43-P; 43 kDa). Western analysis derived from the border zone revealed that Cx43 band pattern is modified qualitatively in response to ischemia/reperfusion. Densitometric analysis of immunoblots revealed similar total amount of Cx43 signals and a reduced intensity of Cx43-P form in tissues undergoing ischemia-reperfusion. Thus, ischemia-reperfusion is associated with progressively reduced phosphorylation of Cx43 from the border zone. The quantitative changes of phosphorylated Cx43 were not significant differences in groups treated with cilostazol (68 ± 15%) compared with data from the control group (63 ± 17% in the control group, P = NS).

Confocal Microscopy

Western data were confirmed by confocal microscopic data analysis. Qualitative immunofluorescent analysis was performed at the border zone. In the sham group, Cx43 antibody produced intense punctate labeling primarily at contacts between cardiomyocytes. After ischemia-reperfusion in the control, there was a loss of antibody immunoreactivity. These alterations in Cx43 immunostaining were similar in animals treated with cilostazol.

五、Discussions

This study demonstrated for the first time that pretreatment (before myocardial ischemia) with cilostazol did not increase the extent of myocardial necrosis after regional ischemia. Altered expression of Cx43 protein by a change in the reduced phosphorylated state was accumulated at the border zone of myocardial infarction especially in dogs treated with either control or troglitazone.

Our results here showed qualitative (reduced phosphorylated) changes of Cx43 protein during ischemia-reperfusion in groups treated with either control or troglitazone, whereas the total amount of Cx43 did not change. The finding was consistent with a recent work [27], showing that infarct size of brain was significantly reduced while gap junctions were blocked in a rat of global cerebral ischemia. Phosphorylation of Cx43 is an important checkpoint for gap junction function. It has been shown that gap junction uncoupling occurs during acute ischemia due to several factors such as a fall in pH_i, an overload of Ca^{2+}, a loss of ATP, an increase in Na^+, and hypoxia [27]. However, the rapid restoration of metabolic disorders after reperfusion results in reopening of gap junctions in surviving cells [27]. Reopening of gap junctions in the presence

of abnormally high intracellular concentration in neighboring cells and cytoskeletal fragility caused by ischemia induced calcium influx and propagation of contraction band necrosis. Transient gap junction blockade during the first minutes of reperfusion until the cell recovers Ca^{2+} control, has been shown to prevent hypercontracture formation [28]. The time sequences of gap junction function can explain, at least in part, reperfusion injury when free radicals massively produce damage. These findings of decreased contraction band necrosis were consistent with our speculation that the major protection of troglitazone is due to inhibition of the development of calcium overload by blocking gap junction functions.

Conclusions

It is concluded that cilostazol, at therapeutic concentrations, did not increase infarct size. The effects of cilostazol appear to be mediated, at least in part, through the reduced phosphorylation of gap junctional protein. This provides perspective for analysis of the pathophysiology and treatment of acute myocardial infarction.

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