Combined Effects of Benidipine and Diltiazem on Cardiohemodynamics in Anesthetized Dogs

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We examined the combined effects of the calcium channel blockers 1,4-dihydropyridine (benidipine) and benzothiazepine (diltiazem) on cardiohemodynamics in anesthetized dogs. Benidipine (3 μg/kg) lowered blood pressure (BP) slightly and continuously increased coronary flow (CF). Diltiazem (300, 1000 μg/kg) decreased BP, heart rate (HR), and the maximum rate of rise of left ventricular pressure (LV dP/dt max) with the increase of doses. Diltiazem increased CF, though it was transient when compared to benidipine. A combination of benidipine (3 μg/kg) and diltiazem (300 μg/kg) showed continuous decreases in BP, HR, and LV dP/dt max, and an increase in CF that was similar to that recorded for the benidipine group. The level of double product (DP: systolic BP×HR, an index of myocardium energy consumption) in the combination group was significantly lower than that of the benidipine group. The plasma concentrations of benidipine and diltiazem in the combination group were similar to those of the groups receiving either drug. These results demonstrate that the combination of benidipine and diltiazem increases CF more continuously than diltiazem alone, and decreases DP more potently than benidipine alone, indicating that the combination therapy possesses favorable properties as a treatment for angina pectoris. Therefore, the combination of benidipine and diltiazem is suggested as a useful treatment for improving the clinical benefits of monotherapy for angina, compared with the use of diltiazem alone at higher doses.

Key words diltiazem; benidipine; angina; combination therapy

Calcium channel blockers are prescribed widely for the treatment of cardiovascular disorders, such as angina pectoris and hypertension. The therapeutic effectiveness of the blockers in these disease states is based on a range of hemodynamic actions, including effects on blood vessels, hearts, and kidneys. Calcium channel blockers are classified into the following three groups on the basis of chemical structures, which show different pharmacological and therapeutic properties: (a) 1,4-dihydropyridines (DHPs), (b) benzothiazepines and (c) phenylalkylamines.1) DHPs exhibit higher selectivity for vasculature than benzothiazepine (diltiazem) and have less effects on nodal tissue of the heart.1) Among the DHPs, benidipine has a high selectivity for vasculature. When the vascular selectivity of various calcium antagonists was evaluated using isolated coronary arteries and right ventricular papillary muscles of dogs, the coronary artery selectivity of benidipine was 14 times higher than that of nifedipine and 19 times higher than that of amlodipine.2,3

In the treatment of angina pectoris, monotherapy using antianginal drugs is generally not completely effective for all types of angina pectoris.4) Combination therapy employing different types of agents has been used in an effort to improve the clinical benefits of monotherapy. We reported recently that the combination of diltiazem and benidipine exerted potent antianginal effects in the vasopressin-induced angina rat model.5) However, there have been no published reports concerning the hemodynamic profile of the combination of diltiazem and benidipine. Therefore, in the present study we examined the hemodynamic effects of diltiazem, benidipine, and the combination of these drugs in anesthetized dogs. In addition, we evaluated the plasma level of the drugs.

MATERIALS AND METHODS

Animals Female Beagle dogs (Nosan Corporation, Yokohama, Japan) weighing 7.7—10.1 kg were used. All animals received humane care in compliance with the ethical standards formulated in the guidelines issued by the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture, No. 141, 1987: “Animal Experiments in Universities etc.,” and the protocol was approved by the Bioethical Committee of the Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd.

Drugs and Chemicals Benidipine hydrochloride was synthesized at Kyowa Hakko Kogyo (Tokyo, Japan). The diltiazem hydrochloride used in animal experiments was purchased from Sigma (St. Louis, MO, U.S.A.). Benidipine was dissolved in physiological saline containing 0.5 vol% Tween 80 (Wako Pure Chemical Industries, Osaka, Japan). Diltiazem was dissolved in physiological saline. The concentration of the drugs was adjusted to yield an injection volume of 0.1 ml/kg. The doses of benidipine (3 μg/kg) and diltiazem (300, 1000 μg/kg) were selected on the basis of the study by Yao and Shirakura.5

For analysis experiments, benidipine-d_{3} as an internal standard (I.S., 96.3% isotopic purity) for the determination of benidipine was synthesized in the Pharmaceutical Research Center, Kyowa Hakko Kogyo. Diltiazem hydrochloride and verapamil hydrochloride as I.S. for the determination of diltiazem hydrochloride were purchased from Wako Pure Chemicals Industries. Oasis® HLB cartridges (30 mg) were obtained from Waters (Milford, MA, U.S.A.). Acetonitrile and Methanol of HPLC grade were obtained from Kanto Chemical (Tokyo).

Experimental Procedure Anesthesia was induced with sodium thiopental (Ravonal®; 25 mg/kg, Tanabe Seiyaku,
Osaka) and maintained by continuous inhalation of halothane (Fluothane®; 1.5—2.5%, Takeda Pharmaceutical, Osaka) after endotracheal intubation. Dogs were ventilated with a volume-cycled respirator using room air (15 ml/kg/stroke, 15 strokes/min). The electrocardiogram (ECG) of standard limb lead II was monitored. Arterial blood pressure was measured with a pressure transducer connected to a polyethylene catheter, inserted into the left brachial artery, and a carrier amplifier (AP-621G; Nihon Kohden, Tokyo, Japan). Polyethylene catheters were inserted into the left brachial vein and the left femoral vein for infusion of test drugs and collection of blood samples, respectively. A catheter transducer (MPC-500, 5F, Millar Instruments, Houston, TX, U.S.A.) was placed into the left ventricle of the heart via the right carotid artery for measurement of left ventricular pressure. The chest was opened by a left lateral thoracotomy, and the heart was suspended in a pericardial cradle. A Doppler flow probe (Transonic Flowprobe®, Transonic System, Ithaca, NY, U.S.A.) was placed on the circumflex branch of the left coronary artery and connected to a pulsed Doppler flowmeter (T101, Transonic System) for the measurement of coronary blood flow. Mean blood pressure (MBP), heart rate (HR), left ventricular dP/dt max (LV dP/dt max) and coronary flow (CF) were calculated and recorded by a real-time analyzing system (HEM Version 3.3, Notocord, Croissy, France). The double product (an index for oxygen consumption) was calculated as the product of HR and systolic BP. After an adequate stabilization period following completion of the surgery, test drugs were injected via the cannula inserted into the left brachial vein. Drug solutions were administered at a volumes of 0.1 ml/kg over a period of about 30 s. Blood samples were collected into standard blood tubes containing heparin, 3, 10, 30, and 60 min after the drug injection in the benidipine at 3 μg/kg, diltiazem at 300 μg/kg, and combination groups. Plasma was separated by centrifugation and frozen at −80°C prior to determination of drug concentration.

Determination of Benidipine Hydrochloride Concentration

The concentrations of benidipine hydrochloride in plasma were measured by liquid chromatography tandem mass spectrometry (API365 LC/MS/MS system, Applied Biosystems/MDS Sciex, Concord, Canada). A mobile phase of 10 mmol/l ammonium acetate buffer (pH 4.0)/acetonitrile (1 : 1) was pumped through a J’sphere ODS-M80 column (75 mm×2.0 mm I.D., YMC, Kyoto, Japan) at a rate of 0.2 ml/min. An aliquot (250 μl) of plasma, 5 μl of I.S. (100 ng/ml methanol solution) and 250 μl of 0.1 mol/l phosphate buffer (pH 8.0) were added into a centrifuge tube. This mixture was vortexed twice with 2 ml of diethyl ether. After centrifugation (1307×g for 5 min), both organic layers were transferred and combined in another tube, and then 20 μl of dimethylacetamide was added to prevent evaporation to dryness which result in loss of analyte due to adsorption to the tube. The combined extracts were evaporated to about 20 μl under a stream of nitrogen, a mobile phase (80 μl) was added and the solution was mixed. An aliquot of sample (25 μl) was injected into the LC/MS/MS.

The calibration curve was linear in the range of 0.02 to 10 ng/ml. Quality control samples spiked with benidipine hydrochloride were prepared and assayed to ensure the accuracy and precision of the method. The intra-day precision and accuracy were 1.2 to 3.3% and within ±13.7% at 0.02, 0.4 and 10 ng/ml with five replicates, respectively. Inter-day precision and accuracy were 1.3 to 5.7% and within ±2.0% at 0.04, 0.8 and 8 ng/ml with a replicate on three separate days, respectively.

Determination of Diltiazem Hydrochloride Concentration

The concentrations of diltiazem hydrochloride in plasma were measured with API365 LC/MS/MS system. A mobile phase of 0.1 vol% acetic acid/methanol/triethylamine (50 : 50 : 0.01) was pumped through a Symmetry C18 column (150 mm×2.1 mm I.D.) at a rate of 0.2 ml/min. Oasis® HLB cartridges were conditioned by elution with 1 ml of methanol and 1 ml of water. A mixture of plasma (100 μl) and 100 μl of I.S. (250 ng/ml solution) were then added and drawn through cartridges. The cartridges were then rinsed with 1 ml water and eluted with 1 ml of acetonitrile/acetic acid (1000 : 1) into test tubes containing 20 μl of dimethylacetamide. The eluates were evaporated under a stream of nitrogen and dissolved with a mobile phase (500 μl). The solutions were centrifuged (ca. 20000×g at 4°C for 10 min) and the supernatant (5 μl) was injected into the LC/MS/MS.

The calibration curve was linear in the range of 10 to 1000 ng/ml. Quality control samples spiked with diltiazem hydrochloride were prepared and assayed to ensure the accuracy and precision of the method. The intra-day precision and accuracy were 2.3 to 2.8% and within ±7.0% at 10, 100 and 1000 ng/ml with five replicates, respectively. Inter-day precision and accuracy were 4.1 to 5.6% and within ±4.0% at 10, 100 and 1000 ng/ml with a replicate on three separate days, respectively.

Statistical Analysis

Values of HR, MBP, LV dP/dt max, and CF are expressed as means±S.E. for 5 dogs in each group. In the group treated with diltiazem at 1000 μg/kg, atrio-ventricular (A-V) block was observed from 8 to 40 min after drug administration in one of five dogs. Data for the other four dogs during the period were used in the analysis. Plasma concentrations of drugs are expressed as means±S.D. All statistics were performed using computer and statistical analysis software (SAS, version 8.2, SAS Institute, Inc., Cary, NC, U.S.A.). Statistic analysis were performed using paired t-test for paired comparisons between pre-values and those for each period, and Student’s t- or Aspin–Welch test for unpaired comparisons. A difference was considered statistically significant at p<0.05.

RESULTS

Figure 1 shows the changes in HR, MBP, LV dP/dt max and CF after i.v. administration of benidipine (3 μg/kg), diltiazem (300, 1000 μg/kg) and a combination of benidipine (3 μg/kg) and diltiazem (300 μg/kg).

Benidipine at 3 μg/kg produced a long-lasting decrease in MBP, and caused slight and transient changes in HR. LV dP/dt max tended to increase, but not significantly. Benidipine increased CF continuously from 1 to 15 min after the administration.

Diltiazem at 300 and 1000 μg/kg decreased HR, and a significant decrease was observed at a dose of 1000 μg/kg. Diltiazem at 300 and 1000 μg/kg caused significant and continuous decreases in MBP and LV dP/dt max with the increased doses. Diltiazem produced only a transient increase in CF. In the group treated with diltiazem at 1000 μg/kg, A-V block...
was observed from 8 to 40 min after the administration in one of the five dogs (data not shown).

The combination of benidipine at 3 μg/kg and diltiazem at 300 μg/kg caused a significant decrease in HR at 10 and 15 min after the administration. The combination decreased MBP, and the peak hypotensive effect was almost the same as the level recorded for diltiazem alone at 300 μg/kg. After 15 min, the hypotensive effect of the combination was almost additive. The combination showed a decrease in LV dP/dt max similar to that recorded for the diltiazem group at 300 μg/kg. The effects of the combination on HR, MBP, and LV dP/dt max were slight when compared with those of diltiazem at 1000 μg/kg. The combination showed a transient increase in CF at 1 and 3 min that was similar to the diltiazem group, and a continuous increase after 5 min that was comparable to the benidipine group. The effects were significant with the exception of values recorded 45 min after the administration.

Figure 2 shows changes in DP in all groups, representing the area under curve (AUC) for changes in DP from 0 to 60 min after drug administration. The combination of benidipine and diltiazem decreased DP, and the effect was more potent than that of benidipine alone. The decrease in DP of the combination group was almost equivalent to that of the diltiazem group at 1000 μg/kg.

Table 1 shows the plasma concentrations of benidipine and diltiazem 3, 10, 30 and 60 min after administration, with or without the other drug. The plasma concentrations of benidipine and diltiazem in the combination group were almost equivalent to those in the groups receiving either drug. The plasma concentrations of each drug were not significantly influenced by its counterpart.

DISCUSSION

Angina pectoris is caused by myocardial ischemia that re-
sults from a decrease in oxygen supply and an increase in oxygen demand. Calcium channel blockers are effective in the treatment of angina pectoris. The calcium channel blockers are thought to improve this oxygen supply-demand imbalance by inducing coronary vasodilation, and by exerting negative chronotropic and inotropic effects. Although these blockers share common mechanisms of action, they differ in their affinity for various tissues in the cardiovascular system. Recently, we reported that the combination of diltiazem and benidipine exerted potent antiangiinal effects in the vasopressin-induced angina rat model. To elucidate the beneficial effects of the combination of benidipine and diltiazem in angina, the present study focused on CF and DP in anesthetized dogs. The results of the present study demonstrate that the combination of benidipine and diltiazem significantly decreased DP in comparison to benidipine alone, and significantly and continuously increased CF. These findings suggest that the combination might possess favorable properties as a treatment for angina pectoris because benidipine exhibits more potent and continuous coronary vasodilating activity than diltiazem, and diltiazem reduces myocardial oxygen demand more effectively than benidipine.

Benidipine is a DHP calcium channel blocker possessing slow-onset and long-lasting vasodilating effects, and it is clinically useful in the treatment of hypertension and angina pectoris. The cohort study reported recently by the Department of Cardiology, Kyushu University, endorsed the results obtained in pre-marketing clinical studies. The Kyusyu University research group began a cohort study in 1981 involving 726 patients receiving vasospastic angina treatment. During that study, various background variables were analyzed, and the effects on prognosis were evaluated for nitrates, beta-blockers, and calcium antagonists, including diltiazem, nifedipine, benidipine and amlodipine. Of these drugs, only benidipine was identified as a drug determining the prognosis of higher doses of diltiazem, but with fewer undesirable side-effects. Nakagawa et al. reported two cases of refractory vasospastic angina that were controlled by the combination therapy of benidipine and diltiazem, while other calcium antagonists, such as amlodipine, nisoldipine, and nifedipine were ineffective in preventing anginal attacks. These results suggest that benidipine may be regarded as one of the drugs that should be tried tested for the treatment of uncontrollable vasospastic angina. However, the precise mechanism of the antiangiinal effects resulting from the combination strategy remains unclear. Further studies will be needed to clarify the significance of this combination therapy in angina pectoris.

In conclusion, the present study showed that the combination of diltiazem and benidipine produced a continuous increase in CF and decrease in DP in anesthetized dogs. These findings suggesting that such an administration strategy may be useful for the treatment of angina pectoris, with fewer un-

![Table 1. Plasma Concentrations of Benidipine and Diltiazem after Intravenous Administration with or without the Other Drug at a Dose of 3 μg/kg for Benidipine and 300 μg/kg for Diltiazem in Anesthetized Dogs](image)
desirable side-effects, in comparison to treatment with diltiazem alone at higher doses.

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