Effects of CV-3611, a New Free Radical Scavenger, on Ischemic Heart Failure in Conscious Beagle Dogs

Masao TANABE and Go KITO

Biology Laboratories, Central Research Division, Takeda Chemical Industries Ltd.,
17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532, Japan

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Abstract—The effects of CV-3611, a new free radical scavenger, on coronary circulation failure and infarct size after ischemia/reperfusion were studied in conscious beagle dogs. The dogs underwent occlusion of the left circumflex coronary artery for 60 min and then were reperfused for 14 days. The dogs were divided into three groups: a control group, a pre-treated group that received CV-3611 or α -tocopherol, and a post-treated group that received CV-3611. During occlusion, varying degrees of ventricular arrhythmia were noted; after reperfusion, the arrhythmia tended to become severe. CV-3611 at a daily dose of 10 mg/kg or 30 mg/kg and α -tocopherol at a daily dose of 60 mg/kg reduced the incidence of overall post-occlusion arrhythmia. Coronary blood flow in the control group was reduced to 20% of the preocclusion level at 7 days after reperfusion, whereas in the CV-3611 and α -tocopherol treated groups, the decreased coronary flow was remarkably suppressed. The infarct size for the CV-3611- and α -tocopherol-treated groups, measured at 14 days after reperfusion, was reduced by 70% when compared with the control group. Based on these observations, it is proposed that CV-3611 exerts its beneficial effects on ischemic tissue by protecting against oxygen free radical-mediated damage induced by ischemia/reperfusion.

Myocardial ischemia caused by coronary artery occlusion initiates a complex sequence of progressively more severe cellular reactions that may lead to cell death and tissue necrosis. A number of factors have been suggested as important determinants of cell death or survival; these include myocardial high energy phosphate depletion (1), intracellular calcium overload (2-4), loss of ionic homeostasis (5), labilization of lysosomes (6), catecholamine (7, 8), and phospholipase-mediated membrane injury (6, 9). Recently, active oxygen species (AOS), including the superoxide anion (O_2^-) and the hydroxy radical ($OH^\cdot$), have been implicated as causal or contributing factors in a variety of types of cell injury, including myocardial ischemia (9-12). Superoxide could induce damage by lipid peroxidation; in oxidizing the membrane lipid, a lipid peroxide radical is formed, and this would act to break down the structure of the membrane (9). Myocardial tissue contains

endogenous scavengers, and under normal conditions, these provide valuable protection against free radical damage. In ischemia, these endogenous scavenging systems are themselves inhibited. These observations have led to the hypothesis that AOS are important mediators of cellular injury, which results from myocardial ischemia and reperfusion. Therefore, the present study was performed to determine the effect of CV-3611 (2-O-octadecylascorbic acid, Fig. 1), a specific

Fig. 1. Chemical structure of CV-3611.

free radical scavenger (13, 14), on myocardial injury after ischemia and reperfusion in conscious dogs.

Materials and Methods

Surgical preparation: Beagle dogs, weighing 11 to 14 kg, were anesthetized with pentobarbital sodium (30 mg/kg, i.v.), intubated, and ventilated with room air using a Harvard respirator. A thoracotomy was aseptically performed at the fourth left intercostal space, and the left circumflex coronary artery (LCX) was dissected free from the adjacent tissue. An electromagnetic flowprobe and a coronary occluder were placed on the LCX. A heparin-filled polyethylene catheter was introduced into the femoral artery to measure the systemic blood pressure. All leads were taken through a subcutaneous tunnel onto the animal's neck and were secured to the skin between the scapulae. After the thoracotomy was closed, the animal was allowed to recover for at least 7 days.

Experimental protocol: While the hemodynamic variables were being measured, the unrestrained dogs were placed in individual metabolic cages in an isolated room. Systemic blood pressure was measured through the cannulated femoral artery with an electrometer (AP-601G, Nihon Kohden). The heart rate was recorded with a cardiograph (AT-601G, Nihon Kohden) triggered by the blood pressure pulse. The coronary artery blood flow was measured with an electromagnetic flowmeter (MFV-2100, Nihon Kohden). These physiologic variables were continuously recorded on a polygraph (RM-6000, Nihon Kohden).

After the baseline values of the hemodynamics and coronary blood flow were measured, the LCX was occluded by pulling a snare for a period of 60 min. After 60 min of ischemia, the flow was restored. All physiologic variables were continuously measured for 5 hr, and at 1, 3, 7 and 14 days after reperfusion. Each animal was randomly assigned to one of three groups: a control group that received no drug, a pre-treated group that was orally given CV-3611 or α -tocopherol at 120 min or 30 min before occlusion and once daily for the next 14 days, and a post-treated group in which CV-3611-treatment was initiated 1

hour after the reperfusion and once daily for the next 14 days. CV-3611 or α -tocopherol was given orally in gelatin capsules.

Measurement of infarct size: After each dog was sacrificed 14 days after reperfusion, its heart was removed. The size of the myocardial infarction was determined using the p-nitroblue tetrazolium staining technique. The left ascending coronary artery and right coronary artery were perfused with 0.5% Evans blue (10 ml). The LCX was perfused with physiologic saline at a constant low pressure (40 mmHg). The area at risk was identified by the lack of Evans blue stain. Infarct size was assessed as a percent of the area at risk.

CV-3611 was synthesized in the Chemistry Laboratories of this Division.

The results were expressed as means $\pm$ S.E.M. and were subjected to one-way analysis of variance followed by Dunnett's test for differences among group means, when necessary.

Results

In all groups, ventricular arrhythmia occurred after occlusion of LCX. Eight of 16 dogs in the control group developed ventricular fibrillation during the period of

Fig. 2. Effect of pretreatment with CV-3611 on the incidence of ventricular arrhythmia after reperfusion in conscious dogs. Open columns indicate the control group. Hatched columns and closed columns indicate the CV-3611-pretreated group. Vertical lines indicate the standard error. CV-3611 was given 120-min before LCX occlusion and then once daily for the next 14 days.

Fig. 3. Effect of post-treatment with CV-3611 on the incidence of ventricular arrhythmia after reperfusion in conscious dogs. Open columns indicate the control group. Hatched columns and closed columns indicate the CV-3611-post-treated group. Vertical lines indicate the standard error. CV-3611 was given initially 60-min after reperfusion and then once daily for the next 14 days.

occlusion and then died. The incidence of ventricular arrhythmia, but not ventricular fibrillation, were noted immediately after reperfusion. In the control group, it gradually increased and peaked 5 hr after reperfusion, and thereafter, a high incidence of ventricular arrhythmia persisted for 24 hr. The incidence of ventricular arrhythmia in the CV-3611 pre- and post-treated groups was less than that in the control group. As shown in Fig. 2, at 5 and 24 hr after reperfusion, ventricular arrhythmia was significantly ($P < 0.05$, compared to control group) lower at a dose of 10 mg/kg of CV-3611 in the pre-treated group, whereas 10 and 30 mg/kg of CV-3611 in the post-treated group significantly inhibited the incidence of reperfusion-induced ventricular arrhythmia at 24 hr (Fig. 3). At an oral dose of 60 mg/kg of α -tocopherol, the incidence of ventricular arrhythmia was significantly lower at 5 and 24 hr after reperfusion (Fig. 4). Ventricular arrhythmia decreased gradually and then disappeared by 7 days in the CV-3611 and α -tocopherol treated group. Furthermore, at 14 days, no ventricular arrhythmia was seen in the control group.

1. Hemodynamic change: The hemodynamic changes after ischemia and reperfusion in the control group are shown in Fig. 5. During occlusion of the LCX for 1 hr, systemic

Fig. 4. Effect of pre-treatment with α -tocopherol on the incidence of ventricular arrhythmia after reperfusion in conscious dogs. Open columns indicate the control group. Hatched columns and closed columns indicate the α -tocopherol-pretreated group. Vertical lines indicate the standard error. α -Tocopherol was given initially 30-min before LCX occlusion and then once daily for the next 14 days.

blood pressure was markedly decreased in the early stage and then tended to return to the pre-occlusion level, whereas the heart rate was slightly decreased. Upon reperfusion, the blood pressure and heart rate decreased again, and thereafter tended to return toward the preocclusion level. The systemic blood pressure recovered to the pre-occlusion value at 3 days after reperfusion. A decrease in

Fig. 5. Hemodynamic changes during LCX occlusion and reperfusion in the control group. BP: blood pressure (open circles indicate mean blood pressure), HR: heart rate, CBF: coronary blood flow.

blood pressure after LCX occlusion in the 10 mg/kg of CV-3611 pre-treated group was less than that in the control group. Furthermore, the systemic blood pressure recovered to the pre-occlusion value at 5 hr in the CV-

3611 (10 mg/kg) pre-treated group, but not in the post-treated group. However, there was no significant difference between the control and the CV-3611- or α -tocopherol-treated group in any of the ischemia/re-

perfusion-induced changes in blood pressure and heart rate observed within the experimental period (Figs. 6, 7 and 8).

2. Blood flow in LCX: Coronary blood flow after reperfusion transiently increased, i.e., reactive hyperemia occurred, and thereafter decreased gradually, resulting in the no-reflow phenomenon 7 days later in 4 of 8 dogs in the control group (Fig. 5). In the CV-3611 pre-treated group, the drug at a dose of 10 mg/kg significantly inhibited reperfusion-induced decrease in coronary blood flow after reperfusion; coronary blood flow at 3 to 14 days after reperfusion was 87 ± 10 to $107 \pm 10\%$ of the initial value (Fig. 6). At 30 mg/kg of CV-3611 in the post-treated group, coronary blood flow at 7 and 14 days after reperfusion was approximately 90% of the

initial value (Fig. 7). At 60 mg/kg of α -tocopherol, coronary blood flow at 7 and 14 days was about 60% of the initial value (Fig. 8).

3. Infarct size: The overall area at risk expressed as a percent of the total left ventricle was $55.7 \pm 3.4\%$ ($n=8$) in the control group, $58.4 \pm 3.8\%$ ($n=10$) in the CV-3611 pre-treated group, $55.5 \pm 2.4\%$ ($n=8$) in the CV-3611 post-treated group, and $55.8 \pm 2.2\%$ ($n=8$) in the α -tocopherol-treated group, indicating that the area at risk did not differ between the control group and the treated groups. In the control group, the mean infarct size was $12.2 \pm 3.0\%$ of the area at risk. At a dose of 10 mg/kg of CV-3611 in the pre-treated group, a dose of 30 mg/kg of CV-3611

Fig. 6. Effect of pretreatment with CV-3611 on mean blood pressure (MBP), heart rate (HR) and blood flow of the LCX coronary artery (CBF) after reperfusion in conscious dogs. The data show the changes in the measured variables expressed as percentage of the initial value. CV-3611 was given 120-min before LCX occlusion and then once daily for the next 14 days.

Fig. 7. Effect of post-treatment with CV-3611 on mean blood pressure (MBP), heart rate (HR) and blood flow of the LCX coronary artery (CBF) after reperfusion in conscious dogs. The data show the changes in the measured variables expressed as percentage of the initial value. CV-3611 was given initially 60-min after reperfusion and then once daily for the next 14 days.

Fig. 8. Effect of pre-treatment with α -tocopherol on mean blood pressure (MBP), heart rate (HR) and blood flow of the LCX coronary artery (CBF) after reperfusion in conscious dogs. The data show the changes in the measured variables expressed as percentage of the initial value. α -Tocopherol was given initially 30-min before LCX occlusion and then once daily for the next 14 days.

in the post-treated group and at a dose of 60 mg/kg of α -tocopherol in the pre-treated group, the infarct size was $3.2 \pm 1.2\%$, $2.4 \pm 1.4\%$ and $2.7 \pm 0.5\%$, respectively (Fig. 9 A, B, C). The mean infarct sizes in both the CV-3611 and α -tocopherol-treated groups were significantly less than the mean infarct size in the control group. However, as shown in Fig. 10, there was a highly significant correlation ($P < 0.05$) between the infarct size and the area at risk. The correlation coefficient, r , was 0.89 in the control group. The linear regression lines for both CV-3611-treated groups (pre-treatment of 10 mg/kg and post-treatment of 30 mg/kg) and the α -tocopherol-group (pre-treatment of 60 mg/kg) were shifted downward, indicating a smaller

Fig. 9. Effect of pre-treatment (A), post-treatment (B) of CV-3611, and pretreatment of α -tocopherol (C) on infarct size after a 60-min occlusion and 14 days reperfusion of the LCX.

Fig. 10. Relation between the extent of the area at risk (LCX/LV, on the abscissa) and infarct size (IS/LCX, on the ordinate) in the control (open circle), CV-3611 (10 mg/kg)-pretreated group (solid circle), CV-3611 (30 mg/kg)-post-treated group (solid triangle) and α -tocopherol (60 mg/kg)-pretreated group (open triangle).

infarct size for the same extent of the area at risk.

Discussion

In this study, varying degrees of ventricular

arrhythmia were noted while the LCX was occluded for 60 min in conscious beagle dogs. After reperfusion, arrhythmia tended to become severe, and then disappeared 7 days later. The LCX blood flow after reperfusion increased transiently and then gradually decreased. Furthermore, the LCX blood flow showed the no-reflow phenomenon in 4 of 8 animals at 7 days after reperfusion. The myocardial infarct size, measured 14 days after reperfusion, was linearly related to the area at risk.

The main consequences of ischemia and reperfusion are cellular injury leading to cell death and disorders of cardiac rhythm. Arrhythmias may lead to cell death in what is otherwise potentially viable tissue. A number of factors might be responsible for the genesis of cellular injury that results from myocardial ischemia and reperfusion; these include disturbances of ionic homeostasis (2–4), stimulation of α - and/or β -adrenergic receptors (15), elevation of intracellular cyclic AMP content (16), the formation and release of lysophosphatides (6, 9, 17), and the metabolism of free fatty acids (18).

Recently, it has been suggested that AOS production may play an important role (19, 20). A few biochemical pathways could be responsible for the generation of AOS at the time of reperfusion of ischemic myocardium. Hypoxanthine and xanthine have been shown to accumulate during myocardial ischemia, as ATP is stepwise degraded (1). Metabolism of hypoxanthine and xanthine is normally catalyzed by xanthine dehydrogenase. However, during ischemia, xanthine dehydrogenase is converted to xanthine oxidase (21). Conversion of hypoxanthine to xanthine and xanthine to uric acid, when catalyzed by the oxidase form of the enzyme, is associated with the generation of superoxide radical (21). Other possible mechanisms of free radical formation include certain steps in prostaglandin synthesis (22) and release of superoxide radicals by phagocytically active neutrophils migrating into ischemically damaged tissue (23). Hydroxyl radical is generated by oxidation of superoxide radical and hydrogen peroxide through the Haber-Weiss and the Fenton reactions (24, 25). The primary cytotoxic effect of oxygen free

radicals may be peroxidation of the polyunsaturated fatty acids of sarcolemmal and subcellular membranes, resulting in formation of lipid peroxides and hydrogen peroxide (26).

The mechanisms underlying oxygen free radical-induced damage in the heart are poorly understood. The heart not only appears to have a much lower concentration of enzymatic defenses against AOS than other tissues (27), but it has become apparent that alterations in the intracellular concentrations of these protective enzymes occur during the development of many seemingly unrelated cardiac pathophysiologic states. A number of recent studies (28–30) have demonstrated that protection of the ischemic myocardium, which undergoes subsequent reperfusion, can be accomplished by supplementing cardiac antioxidant enzymes.

In the present study, we were able to demonstrate that CV-3611 (pre-treatment of 10 mg/kg/day, p.o. and post-treatment of 30 mg/kg/day, p.o.) and α -tocopherol (pre-treatment of 60 mg/kg/day, p.o.), oxygen free radical scavengers, inhibit ventricular arrhythmia, and furthermore showed that two these agents reduce the myocardial infarct size after reperfusion. α -Tocopherol may exert protective effects in hypoxic-perfused and reoxygenated heart (11), and possess antiarrhythmic effects in rats by inhibiting lipid peroxidation (31). CV-3611 is a potent scavenger of AOS, inhibiting lipid peroxidation in rat brain homogenate and linoleic acid peroxidation in micelles initiated by superoxide anion (13). Shimamoto et al. (14) has reported that CV-3611 exerts beneficial effects in alleviating tissue injury due to oxygen free radicals. In addition, the hemodynamic profiles of CV-3611 and α -tocopherol were similar for the control group throughout the course of the experiment, indicating that CV-3611 and α -tocopherol do not limit infarct size by altering myocardial oxygen consumption. These results indicate that the protective effects of CV-3611 and α -tocopherol against myocardial damage in this model may be attributed to its ability to inhibit production of lipid radical.

Furthermore, in this study, we demonstrated that CV-3611 and α -tocopherol

inhibit the decreased LCX blood flow after reperfusion. The coronary artery blood flow after ischemia and reperfusion may be reduced by two primary mechanisms: an increase in vascular resistance caused by ischemia-induced swelling of endothelial cells (32) and vascular compression caused by swelling of myocytes (32). It has been reported that there is a marked increase in cellular swelling with coronary reperfusion after ischemia (33). Severe swelling and lysis of the mitochondria in cardiac myocytes and vascular endothelial cells have been demonstrated in studies of isolated cardiac tissue exposed to solutions capable of generating free radicals (11, 34). Gauduel and Duvelleroy (11) has reported that α -tocopherol inhibits the decreased coronary flow induced by reoxygenation in isolated rat heart and that the protective effect of α -tocopherol may be due to the limiting effect of this antioxidant on lipid peroxides formation. The protective effect of CV-3611 may also be due to the inhibition of lipid peroxidation.

Engler et al. (35) has reported that neutrophils played an important role in the vascular resistance changes during canine ischemia and reperfusion, and that leukocyte depletion resulted in a progressive increase in flow to ischemic myocardium. Although CV-3611 inhibited neutrophil-derived, oxygen free radical-mediated cardiac depression due to intravenous phorbol myristate acetate in anesthetized rats (14), it is not clear whether CV-3611 can reduce neutrophil localization into ischemic tissue or neutrophil activation in this model.

These findings also support the hypothesis that reoxygenation is a major factor in the genesis of reperfusion injury, thereby adding to the myocardial damage caused by ischemia itself.

In summary, the data presented in this report demonstrate that CV-3611 and α -tocopherol limit the extent of irreversible injury arising from myocardial ischemia and reperfusion. We therefore propose that CV-3611 and α -tocopherol exert its beneficial effect by protecting against AOS-mediated damage that occurs as a consequence of the ischemic reperfusion.

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