Cypridina Luciferin Analog Reduces the Incidence of Ischemia/Reperfusion-Induced Ventricular Fibrillation

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ABSTRACT—A Cypridina luciferin analog (CLA), considered to be a sensitive and specific agent for the assay of superoxide, was assessed in isolated hearts for its effects on ischemia/reperfusion injury. Hearts of anesthetized male Wistar rats were isolated and perfused with a modified Krebs-Henseleit bicarbonate buffer to serve as non-recirculating working heart preparations. After 15 min of perfusion to achieve stability, they underwent 20 min of global ischemia and were then reperfused for 30 min with or without 250 μM of CLA, dissolved in the perfusate. The incidence of ventricular fibrillation was only 13% in the CLA group, whereas it was 88% in the controls. The CLA treatment was further associated with significantly increased left ventricular developed pressure and cardiac output; and in contrast, the left ventricular end-diastolic pressure was significantly reduced, as compared with the control group. Thiobarbiturate reacting substance content in the hearts of the CLA group was significantly decreased (27.5±2.4 versus 36.9±9.7 μmol/g dry weight). This study thus indicates that CLA may be useful for alleviating ischemia/reperfusion injury (reperfusion-induced arrhythmia and damage to heart function) involving free radicals.

Keywords: Cypridina luciferin analog, Ischemia/reperfusion injury, Heart (isolated, rat), Lipid peroxide, Free radical scavenger

There have been many reports that myocardial ischemia/reperfusion injury is associated with the production of oxygen-derived free radicals, and that it may be attenuated by oxygen radical scavengers (1, 2). Sources of active oxygen production include the xanthine oxidase enzyme system (3), polymorphonuclear leukocytes (4), the arachidonic acid metabolic system (5), catecholamine metabolism and the mitochondrial electron transport system (6). Administration of various types of free radical scavengers has been tested as a means of therapy for reperfusion injury, but the effects remain controversial (7–10). A Cypridina luciferin analog, 2-methyl-3-phenyl-pyrazin-3-one (denoted below by CLA), displays highly sensitive and specific chemiluminescent reactivity with the active oxygen radicals superoxide anion and singlet oxygen, and it has been considered very useful for assay purposes (11, 12). Since it has the potential to trap superoxide generated after myocardial ischemia followed by reperfusion, we performed the present study to determine whether CLA exerts beneficial effects against the reperfusion injury of ischemic myocardium. In addition, the influence of CLA on lipid peroxidation and high energy phosphate levels was evaluated to gain insight into the mechanisms involved.

MATERIALS AND METHODS

Perfusion

Male Wistar rats (340–390 g) were used for the study. The animals were intraperitoneally injected with 400 IU of heparin and pentobarbital (50 mg/kg); and 5 min later, the hearts were quickly excised. Cardiac arrest was induced by placing the organ in a beaker of ice-cold modified Krebs-Henseleit solution of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, 25.0 mM NaHCO3, 0.5 mM Na2EDTA and 5.5 mM glucose; and each heart was then attached to an aortic cannula by ligatures. Isolated hearts were immediately supported by retrograde perfusion from a reservoir at a height of 75 cm, with an atrial bubble trap 10 cm above the left atrium. The working heart system was then initiated as described previously (13, 14). After 15 min of perfusion, each heart was made globally ischemic by clamping both the aortic candle and
left atrial candle for 20 min. It was then reperfused for another 30 min.

Left ventricular pressure was monitored throughout the protocol using a polyethylene catheter (2.5 French size), which was threaded through the left atrial cannula and introduced into the left ventricle. The aortic, left ventricular, and left atrial pressures and the left ventricular dP/dt were recorded with an RM-6000 polygraph system (Nihon Kohden, Tokyo) via a TP-400T pressure transducer (Nihon Kohden). Aortic cardiac flow was measured with an MFV-3200 electromagnetic flow meter (Nihon Kohden). Electrocardiographic monitoring was carried out via X-150 bipolar electrodes (Nihon Kohden) attached to the right ventricular free wall. The perfusate was modified Krebs-Henseleit bicarbonate buffer (pH 7.40), which was kept at 37°C and bubbled with a 95% O₂ - 5% CO₂ gas mixture.

**Experimental protocol**

CLA was synthesized as described previously (15). The reason for choosing 20 min of ischemia in the present study is that an increasing vulnerability to reperfusion ventricular fibrillation was observed with increasing duration of the preceding period of ischemia as reported by Penny and Sheridan (16). To determine the optimal CLA dose, rat hearts were divided into groups of 6-8 receiving perfusate containing varying doses of CLA from the aortic root over 2 min just before the onset of reperfusion. Subsequent incidences of reperfusion-induced ventricular fibrillation were found to be 88%, 83%, 57%, 13076 and 14% at the doses of 0, 25, 125, 250 and 1250 μM, respectively. Therefore, only the 250 μM CLA dose was chosen for further investigation.

To determine whether administration of this level of CLA exerts a toxic influence under normal conditions, we monitored the heart rate, left ventricular pressure, and peak positive and negative left ventricular dP/dt during the 120 min after single CLA injections, through the left atrial cannula for 2 min, into 5 isolated working heart preparations. CLA at 250 μM had no significant effects on any of the hemodynamic parameters studied, and therefore this dose was concluded to have no adverse influence on cardiac performance.

The sequence of events before ischemia, after ischemia, and during reperfusion were examined by performing experiments on groups of hearts in the following periods: 1) before ischemia, 2) just before the onset of reperfusion, 3) after the first 3 min of reperfusion, and 4) immediately at the end of reperfusion. In addition, the effects of CLA or vehicle (the perfusion medium) were evaluated at these latter two time points.

**Biochemical assays**

At the scheduled time points, each heart was freeze-clamped in a Wollenberger device with liquid nitrogen for the determination of high energy phosphate and lipid peroxide contents. For the ATP and creatine phosphate (CP) assays, a part of each frozen tissue sample was weighed and homogenized with the addition of 2.5 ml of 7% perchloric acid per 100 mg of tissue at 0°C. After deproteinization, tris buffer containing 4 N KOH was added to the homogenate to neutralize the perchloric acid, and then the sample was centrifuged at 1700 x g for 10 min. ATP and CP contents were determined by a modification of the method using McElroy-Strehler’s firefly lantern extract (Sigma, St. Louis, MO, USA); the measurements were read with a bioluminescence reader (Model BLR-101C; Aloka Co., Tokyo), as described earlier (17). In the same way, the CP contents were assayed with the conversion of CP to ATP by adenosine diphosphate and creatine kinase.

For determination of tissue thiobarbiturate reacting substance (TRS), as an indicator of lipid peroxide levels, a part of each frozen tissue sample was homogenized with the addition of 1.15% KCl, H₂SO₄ (1/12 N) and 10% phosphotungstic acid were added to the supernatant and mixed. The TRS content was measured by fluorometric assay of the adduct formed with thiobarbituric acid as described by Yagi (18).

**Statistical analyses**

Statistical analyses were carried out by analysis of variance with Scheffe’s test. P values of less than 0.05 were considered to indicate a significant difference. Results are expressed as means±S.D.

**RESULTS**

**Cardiac function**

No significant differences in any of the preischemia parameters were evident between the two groups of heart preparations (Table 1).

During ischemia, the left ventricular developed pressure (LVDP mmHg) values in both the vehicle-treated and CLA-treated groups showed a marked drop. They recovered gradually during the subsequent reperfusion, with the extent of recovery being significantly higher in the CLA-treated group (see Table 1). After 30 min of reperfusion, the left ventricular end diastolic pressure (LVEDP mmHg) value was markedly elevated in the vehicle-treated group, this increase being largely blocked by CLA. The peak positive left ventricular dP/dt in the CLA-treated group was significantly higher than that in the vehicle-treated group. No difference was found in the peak negative left ventricular dP/dt data.
Table 1. Time-related hemodynamic changes in control hearts and in those treated with CLA

|                | Control Preischemia | Control 30 min reperfusion | CLA Preischemia | CLA 30 min reperfusion |
|----------------|---------------------|----------------------------|-----------------|------------------------|
| HR (l/min)     | 275±40              | 240±57                     | 265±21          | 261±17                 |
| LVDP (mmHg)    | 96±7                | 68±14                      | 101±9           | 91±8*                  |
| LVEDP (mmHg)   | 2.9±0.7             | 12.8±2.6                   | 1.7±1.1         | 3.2±1.2**              |
| LVdP/dt (mmHg/sec) | 3370±440       | 2100±810                   | 3780±630        | 3330±390*              |
| -LVdP/dt (mmHg/sec) | 2190±460       | 1520±560                   | 2420±280        | 2200±560               |
| CO (ml/min)    | 39.8±4.0            | 14.5±9.1                   | 44.9±4.2        | 37.1±1.7**             |
| CE (ml/min)    | 19.8±1.4            | 15.3±3.2                   | 19.1±2.6        | 16.9±1.6               |

Values are means±S.D. for 5 rat hearts. HR, heart rate; LVDP, left ventricular developed pressure (LVsystolic pressure−LVdiastolic pressure); LVEDP, left ventricular end-diastolic pressure; LVdP/dt, peak positive left ventricular dP/dt; −LVdP/dt, peak negative left ventricular dP/dt; CO, cardiac output; CE, amount of coronary effluent. *P<0.05, **P<0.01, compared with the respective control value.

In the vehicle-treated group, the cardiac output (ml/min) after 30 min of reperfusion was significantly lower than that in the group receiving CLA. The coronary sinus flow (ml/min) values, in contrast, showed no essential variation between the two groups (Table 1).

Reperfusion-induced ventricular fibrillation
The optimal dose of CLA was found from the pilot study to be 250 μM. In the group treated with 250 μM of CLA, only 1 of 8 hearts exhibited ventricular fibrillation, this being significantly lower (P<0.05) than the 88% incidence observed in the vehicle-treated group. Even in this one case, a return to normal sinus rhythm was observed within 6 min after the completion of reperfusion. In the vehicle-treated group, in contrast, 7 of 8 hearts demonstrated ventricular fibrillation with an average duration of 13.4±7.1 min (from 5.5 to 26.0 min). Recordings of representative experiments are shown in Fig. 1.

Fig. 1. Representative recordings of ECG, left ventricular pressure (LVP) and left ventricular dP/dt (LVdP/dt) in (a) CLA (250 μM)-treated and (b) vehicle-treated rat hearts. After reperfusion (indicated by an arrow), the ECG tracing of the vehicle-treated hearts showed ventricular tachycardia (VT) followed by ventricular fibrillation (VF); in contrast, that in CLA-treated hearts showed neither VT nor VF.
**Tissue TRS content**

Figure 2 shows the data for myocardial TRS content (μmol/g dry weight) measured as an indicator of lipid peroxidation. Values were 23.4±5.2 and 27.6±9.6, before and after induction of ischemia, respectively. In the vehicle-treated group, TRS content after the first 3 min and at the end of reperfusion were significantly increased at 43.8±1.4 and 36.9±9.7 (μmol/g dry weight), respectively. In contrast, no significant differences were observed between the preischemia TRS content and values after 3 min or 30 min reperfusion in the CLA-treated group. Differences between the CLA-treated and vehicle-treated groups were significant at both time points.

**Tissue ATP and CP content**

Figure 3 summarizes data on the myocardial ATP and CP contents. A significant decline in both ATP and CP contents was observed after ischemia to 58.6% and 45.9%, respectively. In the vehicle-treated group, the ATP level remained at 56.4% and 69.1% of the preischemic value after 3 min and the end of reperfusion, respectively. Addition of CLA was without significant effect. CP promptly recovered, on commencement of reperfusion, exhibiting an "overshoot" after the first 3 min. Myocardial CP content at the end of the reperfusion was approximately equal to that before ischemia. CLA was again without influence.
DISCUSSION

The present study, designed to assess whether myocardial ischemia/reperfusion injury correlates with lipid peroxidation and to evaluate any protective effects of CLA, revealed a significant reduction in reperfusion-induced arrhythmia, dependent on the dose of CLA applied. A single application of 250 μM, which itself proved to be without effects on cardiac function in the perfused heart system, also inhibited the increase in TRS formation caused by reperfusion, that is, protected against tissue lipid peroxidation. This provides direct support for the hypothesis that superoxide may be closely related to the genesis of myocardial defects, in line with earlier findings indicating the involvement of active oxygen radicals in reperfusion injury (2). Previous studies showed that reoxygenation increases lipid peroxides in ischemic myocardium, and suggested that the increase may be related with reperfusion injury (19-22).

Which sources of active oxygen production may play roles in reperfused ischemic myocardium remains controversial, but several authors have described granulocytes to be the main source of superoxide in animals with myocardial ischemia and reperfusion (23). However, granulocytes were not present in our isolated working rat hearts, and they therefore could have exerted no influence on the observed ischemia/reperfusion injury. In the present study, the action of CLA, a derivative of Cypridina luciferin, which displays highly sensitive and specific chemiluminescent reactivity with the active oxygen radicals, superoxide anion and singlet oxygen, and has been found to be applicable for assay purposes (11, 12), was considered dependent on its scavenging capacity. Previous studies reported that various other agents such as tert-butyl-α-phenylnitrone, which is highly reactive with free radicals, decreased the incidence of reperfusion arrhythmia (24). This agent, however, also affected the heart rate and coronary flow, in contrast to the present results where CLA demonstrated neither positive nor negative inotropic effects and had no influence on coronary vascular resistance.

With regard to the timing of scavenger application, the 2 min period immediately previous to the commencement of reperfusion was chosen in the present study as being optimal, given the fact that lipid peroxides only rise rapidly after this time. In earlier studies, agents were administered over a longer time period (8, 24). The present results would suggest, however, that the initial period is the most important. Several authors have shown that the incidence of reperfusion-induced ventricular fibrillation is dependent on the heart rate, with lower rates being protective (26, 27). CLA did not influence the heart rate, indicating that some action other than negative chronotropic effects could have been exerted upon the cardiac muscle by this agent.

The significant improvement in the recovery of left ventricular function observed with CLA after 30 min of reperfusion may be directly due to the reduction of reperfusion injury, and associated with the decrease in the incidence of reperfusion-induced ventricular fibrillation. It was previously reported that high energy phosphate degradation was related to the depression of cardiac function (28), in agreement with our results. While the treatment with CLA preserved cardiac function during reperfusion, there were no significant differences in either ATP or CP contents between the hearts of CLA-treated rats and those of untreated rats during either ischemia or reperfusion in the present study. This indicates that reperfusion injury under the present experimental conditions is independent of tissue energy levels.

The fact that CLA reduced the incidence of reperfusion-induced ventricular fibrillation in association with decreased generation of lipid peroxidation supports the hypothesis that oxygen-derived free radicals play an important role in the genesis of reperfusion-induced arrhythmias. With regard to the underlying mechanism, it is well recognized that free radicals can cause severe membrane injury by initiating some reactions, including lipid peroxidation, which may alter membrane integrity and permeability characteristics (24, 29).

In conclusion, the free radical scavenger CLA, when given immediately before the onset of reperfusion effectively blocked resultant myocardial injury in our isolated working heart system. At the same time, the marked generation of lipid peroxides following myocardial reperfusion was reduced, indicating a causal role for superoxide radicals in myocardial injury. Taking into account the lack of adverse effects on cardiac function, the present results suggest that this agent has the potential for clinical application.

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REFERENCES

1 Nayler, W.G. and Elz, J.S.: Reperfusion injury: laboratory artifact or clinical dilemma? Circulation 74, 215-221 (1986)
2 Werns, S.W., Shea, M.J. and Lucchesi, B.R.: Free radicals and myocardial injury: pharmacologic implications. Circulation 74, 1-5 (1986)
3 Chambers, D.E., Parks, D.A., Patterson, G., Roy, R., McCord, J.M., Yoshida, S., Parmley, L.F. and Downey, J.M.: Xanthine oxidase as a source of free radical damage in myocardial ischemia. J. Mol. Cell. Cardiol. 17, 145-152 (1985)
4 Engler, R. and Covell, J.W.: Granulocytes cause reperfusion ventricular dysfunction after 15-minute ischemia in the dog.
5 O'Brien, P.J. and Hulett, L.G.: Hydroxyl radical involvement in the luminol chemiluminescence from the reaction of arachidonic acid with sheep vesicular gland microsomes. Prostaglandins 19, 683–691 (1980)

6 Otani, H., Tanaka, H., Inoue, T., Umemoto, M., Omoto, K., Tanaka, K., Sato, T., Osako, T., Masuda, A., Nonoyama, A. and Kagawa, T.: In vitro study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury. Circ. Res. 55, 168–175 (1984)

7 Bolli, R., Zhu, W.-X., Hartley, C.J., Michael, L.H., Repine, J.E., Hess, M.L., Kukreja, R.C. and Roberts, R.: Attenuation of dysfunction in the postischemic ‘stunned’ myocardium by dimethylthiourea. Circulation 76, 458–468 (1987)

8 Burton, K.P.: Superoxide dismutase enhances recovery following myocardial ischemia. Am. J. Physiol. 248, H637–H643 (1985)

9 Reimer, K.A. and Jennings, R.B.: Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. Circulation 71, 1069–1075 (1985)

10 Westlin, W. and Mullane, K.: Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? Circulation 77, Supp. I, 1-30–1-39 (1988)

11 Nakano, M., Sugioaka, K., Ushijima, Y. and Goto, T.: Chemiluminescence probe with Cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, for estimating the ability of human granulocytes to generate O2−. Anal. Biochem. 159, 363–369 (1986)

12 Sugioaka K., Nakano, M., Kurashige, S., Akuzawa, Y. and Goto, T.: A chemiluminescence probe with Cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, specific and sensitive for O2− production in phagocytizing macrophages. FEBS Lett. 197, 27–30 (1986)

13 Neely, J.R., Rovetto, M.J., Whitmer, J.T. and Morgan, H.E.: Effects of ischemia on function and metabolism of the isolated working rat heart. Am. J. Physiol. 225, 651–658 (1973)

14 Suzuki, O., Matsubara, T., Kanashiro, M., Nakao, M., Terada, R., Nishimura, H., Haruta, K., Ikeda, T. and Sakamoto, N.: Are diabetic hearts more resistant to ischemia/reperfusion injury? Japan. Circ. J. 57, 328–334 (1993)

15 Inoue, S., Sugioara, S., Kakoi, H. and Goto, T.: Cypridina bioluminescence VI. A new route for the synthesis of cypridina luciferin and its analogs. Tetrahedron Lett. 20, 1609–1610 (1969)

16 Penny, W.J. and Sheridan, D.J.: Arrhythmias and cellular electrophysiological changes during myocardial ‘ischaemia’ and reperfusion. Cardiovasc. Res. 17, 363–372 (1983)

17 Itoh, K., Matsubara, T., Nanki, M., Nishimura, K., Kambe, T., Sugiyma, S., Ozawa, T. and Sakamoto, N.: Relationship between regional myocardial blood flow and tissue ATP content in acute ischemia. Japan. Heart J. 25, 599–608 (1984)

18 Yagi, K.: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. 15, 212–216 (1976)

19 Davies, S.W., Ranjdayalayn, K., Wickens, D.G., Dormandy, T.L. and Timmis, A.D.: Lipid peroxidation associated with successful thrombolysis. Lancet 335, 741–743 (1990)

20 Guarnieri, C., Flamigni, F. and Caldarrera, C.M.: Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J. Mol. Cell. Cardiol. 12, 797–808 (1980)

21 Rao, P.S., Cohen, M.V. and Mueller, H.S.: Production of free radicals and lipid peroxides in early experimental myocardial ischemia. J. Mol. Cell. Cardiol. 15, 713–716 (1983)

22 Roberts, M.J.D., Young, I.S., Trouton, T.G., Trimble, E.R., Kahn, M.M., Webb, S.W., Wilson, C.M., Patterson, G.C. and Adgey, A.A.J.: Transient release of lipid peroxides after coronary artery balloon angioplasty. Lancet 336, 143–145 (1990)

23 Westlin, W. and Mullane, K.M.: Alleviation of myocardial stunning by leukocyte and platelet depletion. Circulation 80, 1828–1836 (1989)

24 Hearse, D.J. and Tosaki, A.: Free radicals and reperfusion-induced arrhythmias: protection by spin trap agent PBN in the rat heart. Circ. Res. 60, 375–383 (1987)

25 Bernier, M., Hearse, D.J. and Manning, A.S.: Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. Circ. Res. 58, 331–340 (1986)

26 Bernier, M., Curtis, M.J. and Hearse, D.J.: Ischemia-induced and reperfusion-induced arrhythmias: importance of heart rate. Am. J. Physiol. 256, H21–H31 (1989)

27 Murdock, D.K., Loeb, J.M., Euler, D.E. and Randall, W.C.: Electrophysiology of coronary reperfusion. A mechanism for reperfusion arrhythmias. Circulation 61, 175–182 (1980)

28 Guth, B.D., Martin, J.F., Heusch, G. and Ross, J., Jr.: Regional myocardial blood flow, function and metabolism using phosphorus-31 nuclear magnetic resonance spectroscopy during ischemia and reperfusion in dogs. J. Am. Coll. Cardiol. 10, 673–681 (1987)

29 Hess, M.L. and Manson, N.H.: Molecular oxygen: Friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. J. Mol. Cell. Cardiol. 16, 969–985 (1984)