

Cyclocreatine Inhibits the Production of Neutrophil Chemotactic Factors from Isolated Hearts

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This study was designed to determine the effect of cyclocreatine on the release of neutrophil chemotactic factors (NCF) from isolated rabbit hearts. We tested the hypothesis that if ischemia is important for the formation of NCF from the myocardium, then blocking (or delaying) ischemic changes with cyclocreatine should inhibit the release of NCF. Two models were used, including (1) perfusion of rabbit hearts (Langendorff apparatus) with oxygenated (95% oxygen) Krebs-Henseleit buffer (K-H buffer) containing 5% cyclocreatine for 120 minutes, and (2) incubating hearts with phosphate-buffered saline (PBS) containing 5% cyclocreatine for 120 minutes. For both models, rabbits were injected intravenously with 10 ml of 5% cyclocreatine solution 30 minutes before the animals were killed and the hearts removed. Control rabbits were injected with 5% creatine solution or saline for 30 minutes before perfusing hearts with K-H buffer or incubating with PBS. Chemotactic activity was assayed in the perfusates and supernatants using modified Boyden chambers and rabbit peritoneal neutrophils as indicator cells. The chemoattractant f-Met-Leu-Phe (f-MLP) was the positive control for a 100% response rate. Isolated hearts perfused with cyclocreatine showed significantly lower chemotactic activity (ie, $1.24 \pm 1\%$ f-MLP; $P < 0.0001$) compared to hearts perfused with K-H buffer ($129 \pm 18\%$) or creatine ($227 \pm 42\%$) (mean \pm standard error). Similar results were obtained using incubated hearts. Next the effect of cyclocreatine on neutrophils in the Boyden chamber

was determined and it was found that it did not alter neutrophil migration, which excludes a direct inhibitory effect on the cells. Furthermore supernatant from cyclocreatine-treated hearts did not inhibit neutrophil chemotaxis to C5a, indicating absence of a chemotaxis inhibitor in this preparation. Results of these studies suggest that the observed low activity recovered in perfusate and supernatant of cyclocreatine-treated hearts is a result of reduction in the synthesis and/or release of the factors from myocardial tissues. Similar to previously established data, cyclocreatine treatment significantly preserved myocardial nucleotide levels (ie, adenosine triphosphate and creatine phosphate), which supports our hypothesis that the formation of NCF is ischemia dependent and that maintaining elevated levels of myocardial energy nucleotides reduced chemotactic factor release. (Am J Pathol 1990, 137:1233-1241)

Neutrophil accumulation has been shown experimentally to occur within the first 1 to 5 hours in myocardium made acutely ischemic by coronary artery occlusion.¹⁻³ These cells also accumulated in ischemic and infarcted myocardium of patients with acute myocardial infarction⁴ as well as in patients who underwent open heart surgery for myocardial revascularization.⁵ The role of neutrophils in postischemic damage to the heart has been the subject of many investigations. Neutrophil infiltration was shown to be associated with 1) capillary no-reflow phenomenon,⁶ 2) ventricular arrhythmias,⁷ and 3) myocardial cell injury.⁸ Rowe et al⁹ showed that canine neutrophils activated *in vivo* with tetradecanoyl phorbol acetate induced significant cardiovascular dysfunction.

In an acute inflammatory reaction, neutrophils constitute the majority of the cells. Chemical signals that activate and recruit neutrophils from the circulation into sites of

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infection or damaged tissue are called neutrophil chemotactic factors (NCF).¹⁰ These factors are also known to increase adhesion of cells to sites of injury and to activate neutrophils to release toxic agents such as oxygen metabolites and proteases.¹¹⁻¹³ In an attempt to understand immunologic mechanisms involved in the recruitment of neutrophils into ischemic myocardium, we investigated the role of myocardial tissues in this process. Interestingly we found that isolated perfused hearts release high levels of NCF.¹⁴⁻¹⁶ The release of these factors from rabbit hearts was detected within the first 5 minutes of perfusion, and it continued for the following 4 hours. Preliminary biochemical characterization revealed that the cardiac-derived factors are proteins of high molecular weight (more than 100 kd), heat labile, water soluble,¹⁴⁻¹⁶ and active *in vivo* by stimulating neutrophil accumulation into rabbit skin. Using high performance liquid chromatography, we showed that these cardiac factors were not similar in chromatographic behavior to either C5a (15 kd), IL-1 (17 kd), or LTB₄ (366 d).¹⁶ The release of NCF from isolated rabbit hearts was not suppressed by the anti-inflammatory drug ibuprofen.¹⁷ We also found that these factors could be recovered from isolated hearts of a number of species, including bovine, rabbit, and porcine,¹⁴⁻¹⁶ as well as human, chicken, and canine (unpublished observations). It is interesting to note that the cardiac-derived factors not only exhibited chemotactic activity but also stimulated neutrophils to release glucosaminidase.¹⁶ Taken together, our studies suggest that the ischemic myocardial tissues themselves participate in the process of cardiac inflammation by releasing factors that recruit neutrophils and activate them to release digestive enzymes, resulting in myocardial cell damage. The extension of this model to other tissues is supported by the results of recent studies, which showed the recovery of NCF from a number of injured tissues, including corneas,^{18,19} stomachs,^{20,21} and vascular systems.^{22,23}

To investigate the role of ischemia in the release of NCF, we explored the use of cyclocreatine, an analog of creatine. A number of studies by Turner and Walker et al²⁴ showed that the addition of cyclocreatine delayed the onset of ischemia in heart,^{25,26} brain,²⁷ and skeletal muscle.²⁸ They further showed that in the cyclocreatine-fed animals, the levels of adenosine triphosphate (ATP) were higher than in controls. They suggested that cyclocreatine provides a protective role of ATP against irreversible cellular damage during ischemic episodes. In the present study, we used cyclocreatine to test the hypothesis that if ischemia is important for the formation of NCF, then blocking (or delaying) its onset with cyclocreatine should inhibit (or delay) the release of NCF. To test this hypothesis, we first determined whether cyclocreatine administration reduces the levels of NCF released from isolated perfused rabbit hearts and, second, we established a simpler model

of incubated rabbit hearts (substituting the perfused model) in an attempt to understand the mechanisms involved in the effects of cyclocreatine on the release of the cardiac factors.

Materials and Methods

Preparation of Cyclocreatine

Cyclocreatine was synthesized in our laboratory according to previous procedures by Griffiths and Walker.²⁹ The purity and the physical characteristics of cyclocreatine was verified using nuclear magnetic resonance imaging.

Experimental Protocol

Adult New Zealand white rabbits (3 to 4 kg) were killed with T-61 (embutanide, mebezonium iodide, tetracaine hydrochloride, benzoic acid [0.14 mg/kg]) administered via the marginal ear vein.¹⁴ Hearts were removed immediately and perfused using the standard Langendorff technique¹⁴ or incubated in beakers. A detailed description of both approaches is presented below.

Perfused Hearts (Beatable Model)

Isolated hearts were perfused with oxygenated (95% oxygen) Krebs-Henseleit buffer (K-H buffer) containing 10 mmol/l (millimolar) dextrose with 3% bovine serum albumin (source, 118 mmol/l sodium chloride, 4.7 mmol/l potassium chloride, 2.5 mmol/l calcium chloride plus 0.5 mmol/l balanced EDTA, 1.2 mmol/l magnesium sulphate, 1.2 mmol/l potassium phosphate, and 25 mmol/l sodium bicarbonate). The perfusion pressure was maintained at 60 mmHg. Myocardial ischemia was induced by circulating a volume of 120 ml of buffer (ie, K-H solution at 37°C) at a reduced coronary flow rate of 12 ml/min.^{14,30} To determine the influence of cyclocreatine on the levels of NCF recovered from isolated hearts, rabbits were injected intravenously (via a marginal ear vein) 30 minutes before they were killed with 10 ml 5% cyclocreatine solution prepared in sterile saline. Control rabbits were injected with 10 ml 5% creatine prepared in saline (Sigma Chemical Co., St. Louis, MO) or saline alone 30 minutes before they were killed. Isolated hearts then were perfused by the K-H buffer alone or containing 5% creatine or 5% cyclocreatine for 2 hours. At the end of 2 hours, the perfusate solutions were collected and assayed for chemotactic activity as undiluted and diluted 1:3 and 1:9 in PBS. A minimum of four rabbit hearts were tested for each described variable.

To control for the cytotoxic effect of cyclocreatine on the myocardium, samples of the perfused hearts (after

120 minutes perfusion) were fixed in 10% buffered formalin and processed for histologic evaluation.¹⁴ We also examined whether cyclocreatine treatment preserves myocardial ATP and creatine phosphate (CP) in perfused rabbit hearts, as previously established by Turner and Walker.^{25,26} As detailed below, samples of perfused hearts (after 120 minutes perfusion) were processed for nucleotide analysis using high performance liquid chromatography.

Incubated Hearts

In an effort to develop a simpler *in vitro* model (compared to isolated perfused hearts), we tested the effect of cyclocreatine on the levels of NCF recovered in supernatants of isolated hearts incubated in beakers with PBS. This model was used further to understand the mechanisms involved in the effects of cyclocreatine on the release of NCF from isolated rabbit hearts.

Rabbits were injected intravenously with 10 ml 5% cyclocreatine (prepared in sterile saline) or saline for 30 minutes before they were killed. Hearts were then removed, washed three times in saline, and incubated in 10 ml freshly prepared PBS (0.1 mol/l [molar] sodium hydroxide, 4 mmol/l sodium diphosphate, and 1 mmol/l sodium monophosphate; pH 7.2) or PBS containing 5% cyclocreatine. Hearts were maintained at 4°C for 2 hours. Supernatant solutions then were collected and assayed (undiluted as well as diluted, 1:3 and 1:9 in PBS) for chemotactic activity using modified Boyden chamber. Samples were stored at 4°C and tested for chemotactic activity within 1 week. No loss of activity was seen at 4°C for 7 days.¹⁴ Freezing significantly decreased activity.¹⁴ A minimum of four rabbits were used for each described variable.

The following studies were performed in an attempt to determine the effect of cyclocreatine on neutrophil migration in the Boyden chambers and whether the drug stimulates the release of chemotactic factor inhibitors from treated hearts. First we assayed for the direct inhibitory effect of cyclocreatine on neutrophil migration stimulated by the chemoattractant C5a. In a 140- μ l well, 75 μ l 5% cyclocreatine solution was incubated with 75 μ l C5a (3ED₅₀) for 30 minutes at 37°C before exposure to neutrophils in the Boyden chamber. If cyclocreatine would exert a direct inhibitory effect on neutrophil chemotaxis, a reduction in cell migration induced by C5a would be expected. Second we assayed for the presence of neutrophil chemotactic inhibitors in supernatant solutions from cyclocreatine-treated hearts. Supernatant solutions (75 μ l) collected from rabbit hearts treated with cyclocreatine were mixed with 75 μ l C5a and incubated for 30 minutes at 37°C before exposure to neutrophils in the Boyden chambers. If an inhibitor(s) for neutrophil chemotaxis is present, a reduction in neutrophil migration induced by

C5a would be expected. Samples were assayed for neutrophil chemotaxis as undiluted and diluted 1:3 and 1:9 in PBS.

Chemotaxis Assay

Chemotaxis assay was performed using modified Boyden chambers, as previously reported by our laboratory.^{14,18} Briefly, rabbit neutrophils were obtained from the peritoneal cavity of white New Zealand rabbits 4 hours after intraperitoneal injection of 300 ml of 0.15 mol/l NaCl containing 0.1% oyster glycogen (Sigma Chemical Co.). The complement component C5a (3ED₅₀) and f-MLP (10⁻⁹ M) (Sigma Chemical Co.) were used as the positive controls for 100% chemotactic response. Hank's balanced salt solution (HBSS) was the negative control for random migration.

Rabbit neutrophils were adjusted to a final density of 2.5 × 10⁶ cells/ml in HBSS containing 0.1% bovine serum albumin (BSA). A 140- μ l amount of neutrophil suspension was placed in the upper compartment of each chamber containing Millipore membrane (Millipore Corporation, Bedford, MA) of porosity 8 μ m. The lower compartment of the chamber contained 150 μ l of C5a, f-MLP, HBSS, heart perfusates, and supernatant solutions from rabbit hearts. Chambers then were incubated for 1 hour at 37°C in 5% CO₂/95% air atmosphere to permit cell migration across the membrane. After incubation, filters were removed, fixed (100% ethanol), stained (with 100% hematoxylin), and mounted on glass slides for counting using an Optomax Image Analyzer (Optomax, Burlington, MA) of high-power fields (40X).

Cell migration in response to the putative chemoattractants was expressed as a percentage of maximum chemotactic response by f-MLP or C5a. Chemotactic index is defined as distance traveled in the filter × cell number

Neutrophil chemotactic activity (% f-MLP)

$$= \frac{\text{C}^{\text{perfusate or supernate}} - \text{C}^{\text{HBSS}}}{\text{C}^{\text{C5a or f-MLP}} - \text{C}^{\text{HBSS}}} \times 100$$

All data were expressed as mean value ± standard error. Student's unpaired *t*-test was used for statistical analysis. A minimum of two chemotaxis experiments were performed for each collected perfusate or supernate. Each sample was assayed in duplicate (ie, using two filters) and cells that migrated within the filters were counted in six different areas.

Assay for Nucleotides

The ability of cyclocreatine treatment to preserve myocardial ATP levels in response to ischemia was previously demonstrated by Turner and Walker.^{25,26} Therefore we

Table 1. *Effects of Cyclocreatine on High-energy Phosphate Levels in Perfused Rabbit Hearts*

Hearts	CP	%	ATP	%
Nonischemic control*	10.49 ± 0.51	100	3.90 ± 0.40	100
Buffer	0.10 ± 0.01	2.6	0.10 ± 0.01	1.0
Creatine	0.18 ± 0.03	6.7	0.26 ± 0.05	1.7
Cyclocreatine	4.07 ± 0.36	28	1.10 ± 0.08	39

* Hearts isolated from rabbits were processed immediately to determine nucleotide levels.

Isolated rabbit hearts preinfused with cyclocreatine, creatine, or buffer were then perfused with the same agents for 120 minutes as described in the Experimental Protocol. Heart biopsies were extracted and assayed on high-pressure liquid chromatography, as described in the Experimental Protocol. CP and ATP results ($\mu\text{mol/g}$ wet weight) are expressed as mean \pm SE with n = minimum of six biopsies.

used a limited number of rabbits to determine whether cyclocreatine also would preserve myocardial ATP and CP in hearts perfused for 120 minutes. For these studies, rabbits were injected intravenously with 5% cyclocreatine, 5% creatine, or saline 30 minutes for they were killed. Hearts then were removed and perfused with oxygenated cyclocreatine, creatine, or K-H buffer, as described before. After 120 minutes, heart samples were immersed immediately in liquid nitrogen and stored at -70°C until extracted. From each of two hearts, three biopsy specimens were homogenized in 6% perchloric acid using a polytron tissue homogenizer (Brinkman Instruments, Westbury, NY), centrifuged, neutralized to pH 7.0 with 5 mol/l potassium carbonate, and filtered for assay on high-pressure liquid chromatography, as described by Cordis et al.³¹

Running each extract twice, a volume of 25 μl of a filtered and neutralized perchloric acid extract was injected onto a Nova-Pack C_{18} column with a degassed initial mobile phase of 48 mmol/l monobasic potassium phosphate, 1 mmol/l tetrabutylammonium phosphate (pH 5.8, with potassium hydroxide) at a flow rate of 2 ml/minute. Using a Waters Associates Model 490 programmable multi-wavelength ultraviolet detector (Water Associates, Milford, MA), the effluent was monitored at 210 nm for 4 minutes to measure CP and then monitored at 259 nm for 6 minutes to measure ATP. The initial mobile phase was used for 4 minutes, followed by a step gradient to 20% acetonitrile in the degassed initial buffer. After the 10-minute run, the column was equilibrated with the initial mobile phase for 25 minutes before the next injection. The ultraviolet detector was set at zero on injection and during the change in wavelength. A blank was run initially and used for baseline subtraction.

A calibration curve was produced by using MAXIMA 820 software (Waters) system for each of the high-energy phosphate compounds. Using external standards, six different concentrations ranging from 10 $\mu\text{mol/l}$ [micromolar] to 1 mmol/l were used. A response factor then was calculated from the slope of each curve.

Results

Effects of Cyclocreatine on Myocardial Cells

The toxic effects of cyclocreatine administration on myocardial cell morphology was compared to control hearts

perfused with creatine or K-H buffer for 120 minutes. Control hearts showed patches of eosinophilic degeneration of myocardial fiber cytoplasm characteristic of early ischemia in myocardium. Patches of contraction bands associated with ischemia also were evident. Cyclocreatine-treated hearts, on the other hand, showed only occasional small foci of contraction bands and no significant eosinophilic changes (data not shown). These results exclude a damaging effect of cyclocreatine on myocardial cells.

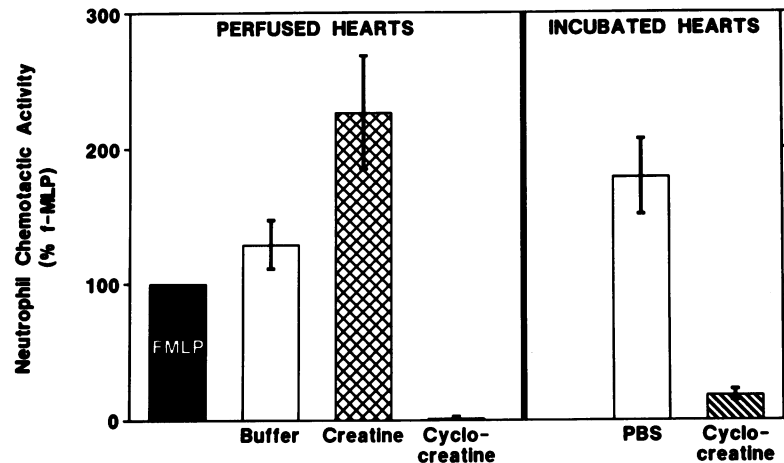
Effects of Cyclocreatine on Myocardial Nucleotide Levels

As previously established by Turner and Walker,^{25,26} our studies showed that hearts perfused with cyclocreatine for 120 minutes maintained almost 40% of the ATP and 30% of the CP of normal nonischemic levels (Table 1). Control hearts perfused with creatine or K-H buffer, on the other hand, showed a loss of more than 95% for both ATP and CP. The preservation of ATP and CP in cyclocreatine-treated hearts and not in control groups supports its known functional response in our model of isolated perfused hearts.

Effects of Cyclocreatine on the Generation of Neutrophil Chemotactic Factors from Isolated Hearts

Having established that cyclocreatine 1) does not induce myocardial cell injury and 2) functions in our system as previously established by preserving myocardial nucleotides, we investigated the effects of cyclocreatine on the levels of NCF recovered in perfusates and supernatants of isolated rabbit hearts. As indicated in Figure 1, chemotactic activity recovered in the perfusate of hearts treated with cyclocreatine for 120 minutes was significantly lower (1% of the response obtained with the positive control, f-MLP) compared to activity detected in solutions of hearts perfused with either creatine (227%, $P < 0.0001$) or K-H buffer (129%, $P < 0.0001$). Similar findings were observed for hearts incubated in beakers with cyclocreatine (18%) or PBS (179%) ($P < 0.0001$) for 120 minutes

Figure 1. Neutrophil chemotactic activity recovered in perfusates and supernates of control (buffer, creatine, or PBS) and cyclocreatine-treated hearts. Rabbits were injected intravenously with 5% cyclocreatine, 5% creatine, or saline 30 minutes before being killed. Hearts were removed and perfused using the Langendorff technique or incubated in a beaker for 120 minutes with the above-described solutions. A minimum of four rabbit hearts were used for each variable.

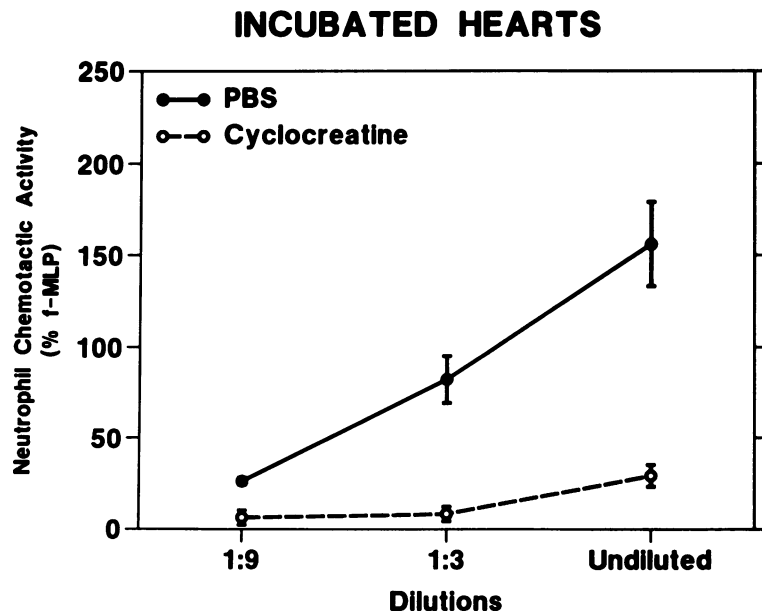


(Figure 1). Because both models (ie, perfused and incubated) showed a similar response (ie, reduction in chemotactic activity following cyclocreatine treatment), the beaker model was used to elucidate further a possible mechanism of action for cyclocreatine. As indicated in Figure 1, creatine-treated hearts showed marked chemotactic activity (227%) compared to its analogue cyclocreatine (1.24%). The toxic effect of creatine on myocardial tissues needs further investigation.

To determine whether the reduced response in chemotaxis seen in the cyclocreatine-treated hearts is due to a desensitization effect resulting from an elevated level of chemoattractants in the preparation, we tested lower concentrations of supernatant solutions. Figure 2 shows a decline in chemotactic activity in both PBS and cyclocreatine-treated hearts (and not an increased response)

when samples were diluted 1:3 and 1:9, indicating that the observed reduction in activity of cyclocreatine samples is not due to a desensitization response on neutrophils. We also investigated whether the observed reduction in chemotactic activity recovered in solutions of cyclocreatine-treated hearts is due to 1) direct inhibitory effect of cyclocreatine on neutrophil migration, and/or 2) the presence of a chemotactic factor inhibitor. Results showed that cyclocreatine does not significantly reduce neutrophil migration stimulated by C5a (Figure 3), indicating that the reduction in activity is not due to a direct inhibition of cyclocreatine on neutrophil migration. We have shown also that incubation of supernatant solutions obtained from cyclocreatine-treated hearts with C5a in the Boyden chamber did not reduce chemotactic activity induced by C5a alone (Figure 4), indicating absence of a chemotaxis

Figure 2. Dose-response curve of chemotactic activity recovered in supernatant solutions of control (PBS) ($n = 1$) and cyclocreatine-treated ($n = 1$) hearts (a representative of four experiments). Rabbits were injected intravenously with 5% cyclocreatine or saline 30 minutes before being killed. Hearts were removed and incubated in a beaker for 120 minutes with cyclocreatine or PBS. Samples were assayed as undiluted and diluted 1:3 and 1:9 in PBS.



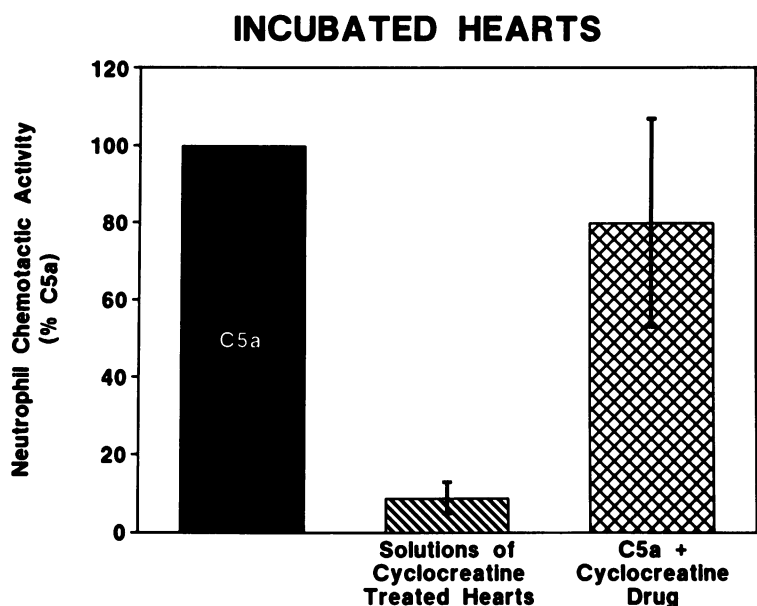


Figure 3. Effects of cyclocreatine on neutrophil migration stimulated by C5a in the Boyden chamber (average of three experiments). Note the absence of significant reduction in neutrophil chemotaxis induced by the chemoattractant C5a following mixing with cyclocreatine, indicating lack of a direct inhibitory effect of the drug on neutrophil migrations.

inhibitor. These results suggest that cyclocreatine treatment reduced the levels of NCF recovered in supernatant solutions by inhibiting the release and/or synthesis of the factors from myocardial tissues.

Effects of Time of Cyclocreatine Administration

In this study, we examined whether preinfusion of cyclocreatine for 30 minutes before the onset of ischemia is necessary for the observed reduction in NCF levels and preservation of nucleotides. In a limited study (two rabbits), hearts were isolated and perfused with buffer containing 5% cyclocreatine for 120 minutes without preinfusion with the drug. Neutrophil chemotactic activity recovered in the perfusate solutions was 105 ± 15 of f-MLP. Hearts extracted for nucleotides showed low levels of both ATP ($0.20 \pm 0.04 \mu\text{mol/g}$ wet weight – 5.1% of nonischemic control) and CP ($1.24 \pm 0.22 \mu\text{mol/g}$ wet weight – 11.8% of nonischemic control). These results indicate the necessity of preinfusing cyclocreatine into nonischemic healthy hearts for the full effect of the drug to be seen.

Discussion

Studies reported here indicated the significant reduction in neutrophil chemotactic activity recovered in perfusate and supernatant solutions of isolated rabbit hearts following treatment with cyclocreatine. In an effort to understand the mechanisms involved in the observed reduction in activity, we examined whether the drug 1) directly inhibited

neutrophil migration, 2) stimulated the release of inhibitors for neutrophil chemotaxis, and/or 3) blocked the synthesis and release of NCF from the myocardium. The possibility that cyclocreatine interfered with neutrophils in the chemotactic chambers is ruled out by results that showed that cyclocreatine did not inhibit cell migration stimulated by the chemoattractant C5a. Similarly supernatant from cyclocreatine-treated hearts did not inhibit neutrophil chemotaxis stimulated by C5a, indicating absence of a chemotaxis inhibitor. Further studies are needed to determine whether cyclocreatine treatment does not stimulate the release of chemotactic factor inhibitors from ischemic myocardium. The ruling out of these two possibilities suggests that the observed reduction in chemotactic activity following cyclocreatine treatment is due to drug interference with the formation of NCF. Future studies aimed at purifying the cardiac-derived NCF and the development of an enzyme-linked immunospecific assay will determine directly whether cyclocreatine treatment interferes with the synthesis and release of the factors. Additional studies using blockers for protein synthesis and exocytosis also will enhance our understanding for the possible role of membrane stabilization and/or protein synthesis in the formation of chemotactic factors.

Because cyclocreatine administration is known to elevate the levels of ATP and CP in ischemic hearts,^{25,26} we determined the levels of both nucleotides in isolated rabbit hearts perfused for 120 minutes with cyclocreatine, creatine, or buffer. Cyclocreatine treatment maintained significantly high levels of ATP and CP compared to control ischemic hearts (ie, creatine and buffer). As previously established by Turner and Walker,^{25,26} our studies showed that hearts perfused with cyclocreatine for 120 minutes

INCUBATED HEARTS

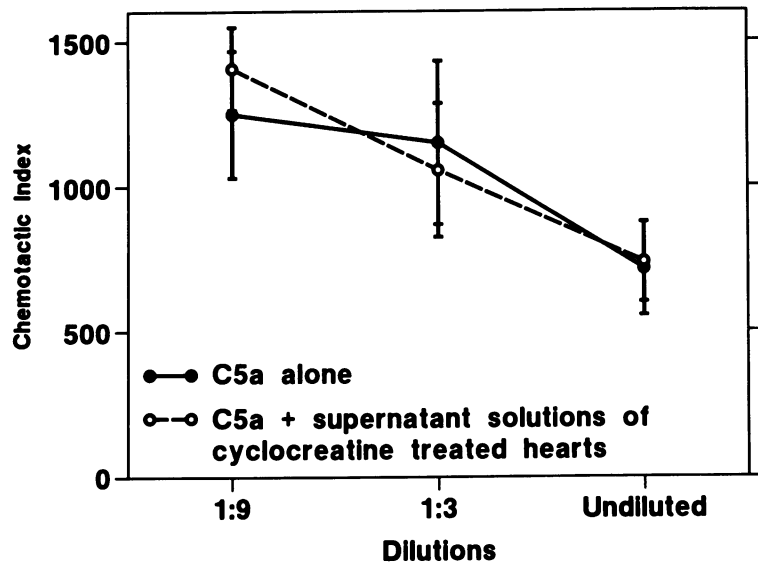


Figure 4. Effects of supernatant solutions collected from a cyclocreatine-treated heart on the neutrophil migration stimulated by C5a (a representative of three experiments). A rabbit was injected intravenously with 5% cyclocreatine for 30 minutes before being killed. Hearts were removed and incubated in a cyclocreatine solution for 120 minutes. Supernatant solutions of these hearts were mixed with C5a before assaying for neutrophil chemotaxis. Note the lack of reduction of neutrophil chemotaxis induced by the mixture of cyclocreatine solution and C5a compared to C5a alone, indicating absence of inhibitors in the cardiac preparation.

maintained almost 40% of the ATP and 30% of the CP of normal nonischemic levels. Studies by Turner and Walker^{25,26} also suggested that cyclocreatine provides a protective role of ATP against irreversible cellular damage during ischemic episodes. Whether preservation of ATP and CP by cyclocreatine during ischemia results in less cell damage and consequently reduction in the recovered levels of NCF is a possibility that must be studied. Results reported here, therefore, support the hypothesis that isolated hearts release NCF and that cyclocreatine administration reduced the levels of the recovered chemotactic activity, presumably by maintaining high energy phosphate levels.

Studies by Turner and Walker^{25,26} also indicated that for cyclocreatine to delay effectively the depletion of ATP during myocardial ischemia, it must be absorbed in advance (ie, hours to days) by healthy nonischemic hearts. Interestingly we found that isolated rabbit hearts perfused with cyclocreatine without previous intravenous injection of cyclocreatine did not reduce the levels of NCF recovered in perfusate solutions, nor did it maintain high energy levels (ie, ATP and CP). These results support the need for healthy nonischemic hearts to use cyclocreatine for maintaining elevated levels of nucleotides and reducing chemotactic activity. These results also suggest that ATP generated by CP (constituting 10% of the cell ATP) plays a role in the appearance of chemotactic factors after ischemia because a drop in ATP levels results in release of chemoattractants.

The role of neutrophils in postischemic damage to the heart has been well documented. Neutrophil infiltration was shown to be associated with 1) capillary no-reflow phenomena,⁶ 2) ventricular arrhythmia,⁷ and 3) the ob-

served myocardial damage^{8,32} by releasing destructive enzymes such as collagenase, elastase, and cathepsin^{12,33} and free oxygen radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radicals.^{13,34,35} Rowe et al⁹ showed that canine neutrophils activated with tetradecanoyl phorbol acetate induced significant cardiovascular dysfunction.

A number of agents that reduce neutrophil infiltration and function have been tested for their effects on modulating myocardial injury. Ibuprofen,³⁶⁻³⁸ BW755C39,^{39,40} dipyridamole,⁴¹ and superoxide dismutase⁴² were shown to suppress neutrophil accumulation in inflammatory lesions and to exert cardioprotective effects. Neutrophil depletion with either filtration,⁴³ specific antineutrophil antiserum,⁴⁴ or administration of hydroxyurea⁴⁵ confirmed that myocardial damage is reduced when neutrophils were prevented from invading ischemic myocardium. These studies provide evidence for the active participation of neutrophils in exacerbating acute myocardial ischemia and infarction.

In summary, we found that cyclocreatine administration reduces the levels of NCF recovered in perfusate and supernate solutions of isolated rabbit hearts. Reducing the inflammatory mediators results in less neutrophil accumulation into myocardial tissues after ischemia, with consequent protection from neutrophil-associated ventricular arrhythmia and myocardial cell damage as well as ischemia due to plugging capillaries.

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