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Anti-ischemia/reperfusion of C1 inhibitor in myocardial cell injury via regulation of local myocardial C3 activity

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Abstract

C3 is common to all pathways of complement activation augmenting ischemia/reperfusion (I/R)-induced myocardial injury and cardiac dysfunction. Complement inhibition with the complement regulatory protein, C1 inhibitor (C1INH), obviously exerts cardioprotective effects. Here, we examine whether C1INH regulates C3 activity in the ischemic myocardial tissue. C1INH markedly suppressed C3 mRNA expression and protein synthesis in both a model of I/R-induced rat acute myocardial infarction (AMI) and the cultured rat H9c2 heart myocytes. At least, this regulation was at the transcriptional level in response to oxygen tension. *In vitro*, C3 deposition on, and binding to, the surface of rat myocardial cells were significantly blocked by C1INH treatment. C1INH could inhibit classical complement-mediated cell lysis via suppressing the biological activity of C3. Therefore, C1INH, in addition to inhibition of the systemic complement activation, prevents myocardial cell injury via a direct inhibitory role in the local myocardial C3 activity.

Keywords: Complement; C1 inhibitor; C3; Hemolysis; Cardiomyocyte; Ischemia; Reperfusion

Although reperfusion of the ischemic myocardium is an absolute necessity to recover tissues from final death, accumulating data indicate that reperfusion itself contributes to additional myocardial cell injury, and this injury occurs when blood flow is restored after an extended period of ischemia [1,2]. I/R is a potent activator of complement components [3–5]. Clinical and experimental studies using several organs have shown that reperfusion after ischemia results in local complement activation leading to the production of chemotactic factors [6,7], indicating that complement protein expression in local organs may be involved in complement activation. Blocking the complement activation with C1INH appears to be an effective way of preventing ischemic cardiomyocyte from injury following reperfusion [8–10]. Complement inhibition at the level of C3 reduces myocardial infarct size [11]. Using a model of I/R-induced AMI in rat and *in vitro* hypoxia/reoxygenation-induced cultured H9c2 rat heart myocytes, we investigated whether C1INH has a direct effect on regulating myocardial C3 activity. These data demonstrated that C1INH, in addition to inhibition of the complement system activation, protects from myocardial cell injury via a direct regulation in local myocardial C3 activity.

Materials and methods

I/R-induced AMI model in rat. Sprague–Dawley rats (200–250 g, male or female randomly, 8 weeks) were randomly divided into the following

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groups: (1) sham group ($n = 5$); (2) I/R (reperfusion at 3 h or 72 h after ischemia at 30 min) rats receiving 0.9% NaCl (10 ml/kg) intravenously before surgery ($n = 5$); and (3) I/R (reperfusion at 3 h or 72 h after ischemia at 30 min) rats treated with C1INH (40 U/kg, 1 U ≈ 0.15 mg, Behring Company, Marburg, Germany) intravenously before surgery ($n = 5$).

Cell culture. H9c2 rat heart myocytes (ATCC, Rockville, MD) were cultured in monolayers in DMEM with 10% FBS, glutamine (2 mmol/l), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in normoxic condition (5% CO_2, 21% O_2, and 74% N_2) in a humidified incubator at 37 °C and used for all experiments at ~70% confluence. Rat H9c2 heart myocytes were grown to 70% confluence, changed to fresh medium, and transferred to a triple gas incubator with hypoxia (5% CO_2, 1% O_2, and 94% N_2). After hypoxia, these cells were recovered with reoxygenation (5% CO_2, 21% O_2, and 74% N_2). These cells induced by hypoxia/reoxygenation were treated with or without C1INH.

Immunohistochemical staining. The sections were incubated with mouse anti-monoclonal C3 (200 μg/ml, Santa Cruz Biotech, USA) at 1:50 dilution overnight at room temperature. After washing with PBS, the sections were incubated with the horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Pierce Biotechnology, Rockford, IL) and finally developed using 3,3 *o*-diaminobenzidine. The sections were examined with an Olympus light microscope.

Western blot. Total protein (50 μg/15 μl) was run and then transferred to a nitrocellulose filter. Membranes were subsequently exposed to mouse anti-monoclonal C3 at 1:200 dilution at 4 °C for 12 h. Bound antibody was detected by the HRP-conjugated anti-mouse IgG antibody. Finally, enhanced chemiluminescence (ECL) detection reagents (KPL, Washington, USA) were employed to visualize the peroxidase reaction products. The density of the band was measured by a Bioimaging Analyzer (scanned on UMAX MagicScan and analyzed with the software package HPIAS-1000, Olympus, Japan).

RT-PCR. C3 mRNA was detected with primers: 5′-CTACCCCT TACCCCTCACTCCTTCCACCTT-3′ and 5′-ATTCCTTACTGGCTG GAATCTTGATGAAGA-3′ by RT-PCR. GAPDH with primers: 5′-TCC CTCAAGATTGTCAGCAA-3′ and 5′-AGATCCACAACGGATAC ATT-3′ and β-actin with primers: 5′-TGGATGACGATATCGC TGC-3′ and 5′-AGGGTCAGGATACCTCTCTT-3′ were used as the controls. RT-PCRs were performed with 1 μg of total RNA, followed by 40 cycles of PCR amplification. The density of each PCR band was analyzed with a densitometer (ImageMaster VDS, Vilber Lourmat, Germany).

Northern blot. C3 mRNA was detected using antisense oligonucleotides labeled with [γ-^{32}P]ATP (6000 Ci/mmol). Pre-hybridization (3 h) and hybridization overnight with ^{32}P-labeled oligonucleotide probe were carried out at the hybridization temperature. Antisense oligonucleotide probes were as follows: rat complement C3: 5′-GTCCAGGATGGACAT AGTAGCATCCACG-3′; and rat β-actin: 5′-GTGTGGTGCCAAATCT TGTCCATATC-3′. Autoradiographs were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In vitro C3 deposition assay. C3 deposition was measured as previously described [12,13]. Rat H9c2 heart myocytes (1×10^5) were added to each well: (1) 20% human serum (HS), (2) 20% HS + 150 μg/ml C1INH, and (3) 150 μg/ml C1INH. The cells were subjected to normoxia or 24 h of hypoxia or 2 h of reoxygenation after 24 h of hypoxia. The cells were incubated with polyclonal goat anti-human C3 antibody (Cappel, West Chester, PA) for 1 h at room temperature. The plates were incubated with HRP-conjugated anti-goat IgG antibody. *o*-Phenylenediamine dihydrochloride substrate was added and the color reactions were developed for 5 min at room temperature and terminated with 3 N HCl. The absorbances (405 nm) were measured using a microtiter plate reader.

In vitro C3 binding assay. Rat H9c2 heart myocytes (1×10^5) were incubated with one of the following: (1) C3 (150 μg/ml), (2) C1INH (150 μg/ml), and (3) C3 (150 μg/ml) + C1INH (150 μg/ml) in fresh media without serum. The cells were subjected to normoxia or 24 h of hypoxia or 2 h of reoxygenation after 24 h of hypoxia. The cells were incubated with polyclonal goat anti-human C3 antibody and polyclonal rabbit anti-human C1INH antibody for 1 h at room temperature. The plates were incubated with HRP- anti-goat IgG antibody or anti-rabbit IgG antibody. *o*-Phenylenediamine dihydrochloride substrate was added and the color reactions were developed for 5 min at room temperature and terminated with 3 N HCl. The absorbances (409 nm) were measured using a microtiter plate reader.

Rat C3-deficient serum preparation and hemolysis. Rat sera were incubated with mouse anti-C3 monoclonal antibody (50 μg/ml) at 4 °C for overnight and then added with anti-mouse IgG (Sigma–Aldrich, St. Louis, MO) at room temperature for 1 h. The purified sera were detected by a solid-phase capture sandwich ELISA as compared with standard human plasma ($1/10^4$ dilution). C3 could not be detected in the sera. GVB^{2+} buffer was added in triplicate to a 96-well plate. Rat C3-deficient serum was diluted to 40% vol/vol with GVB^{2+} buffer and added to the rows of the same 96-well plate such that the final concentration of rat serum in each well was 20%. The myocyte supernatant was obtained from lysed extracts of rat H9c2 heart myocytes induced by hypoxia/reoxygenation in the presence or absence of C1INH (150 μg/ml). A solid-phase capture sandwich ELISA was used to compare with standard human plasma ($1/10^4$ dilution). C1INH could not be detected in the supernatant preparation. The plate was incubated at room temperature for ~30 min. Chicken erythrocytes (5×10^7) in GVB^{2+} buffer (4 ml) were sensitized with anti-rat erythrocyte polyclonal antibody (Intercell Technologies, 0.1% vol/vol) and incubated at 4 °C for 15 min. The cells were washed two times with GVB^{2+} buffer and resuspended to a final volume of 2.4 ml in GVB^{2+} buffer. The erythrocytes (30 μl/well, 2×10^6 cells) were added to the plate containing serum and the myocyte supernatant (40%, 20%, and 10%) as described above, mixed well, and incubated at 37 °C for 30 min. The plate was then centrifuged at 1000*g* for 2 min, and 85 μl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at 415 nm with a microplate reader. Hemolysis (%) = 100 × [(OD sample) − (OD GVB^{2+} control)]/[(OD 100% lysed control) − (OD GVB^{2+} control)].

Statistical analysis. Statistical significance was estimated by one-way analysis of variance (ANOVA) and data were analyzed using the Tukey Multiple Comparisons Test (GraphPad Software Inc., San Diego, CA). Representative result is shown for three experiments.

Results

C1INH reduces myocardial C3 in rat AMI

We investigated the effects of C1INH on C3 action in ischemic myocardium during rat AMI. As illustrated by immunostaining analysis on sections from free wall of the myocardial ischemic area, C3 protein staining was increased in ischemic myocardial tissue at 3 h and 72 h, but such increases were not observed by the treatment with C1INH (Fig. 1A). To determine whether the increase of myocardial C3 was derived from plasma or from local synthesis of ischemic myocardial cells, cytoplasmic C3 mRNA isolated from the ischemic myocardial cells was analyzed by RT-PCR. The results showed that I/R induced cytoplasmic C3 mRNA was expressed in these cells. Administration of C1INH completely inhibited this induction at 3 h ($P < 0.01$) and 72 h ($P < 0.01$) as compared with the sham control group (Fig. 1B). Western blotting analysis also showed the similar results (Fig. 1C).

C1INH suppresses hypoxia/reoxygenation-mediated C3 in rat heart myocytes

We further investigated the effect of C1INH on the increases of cytoplasmic C3 mRNA expression and

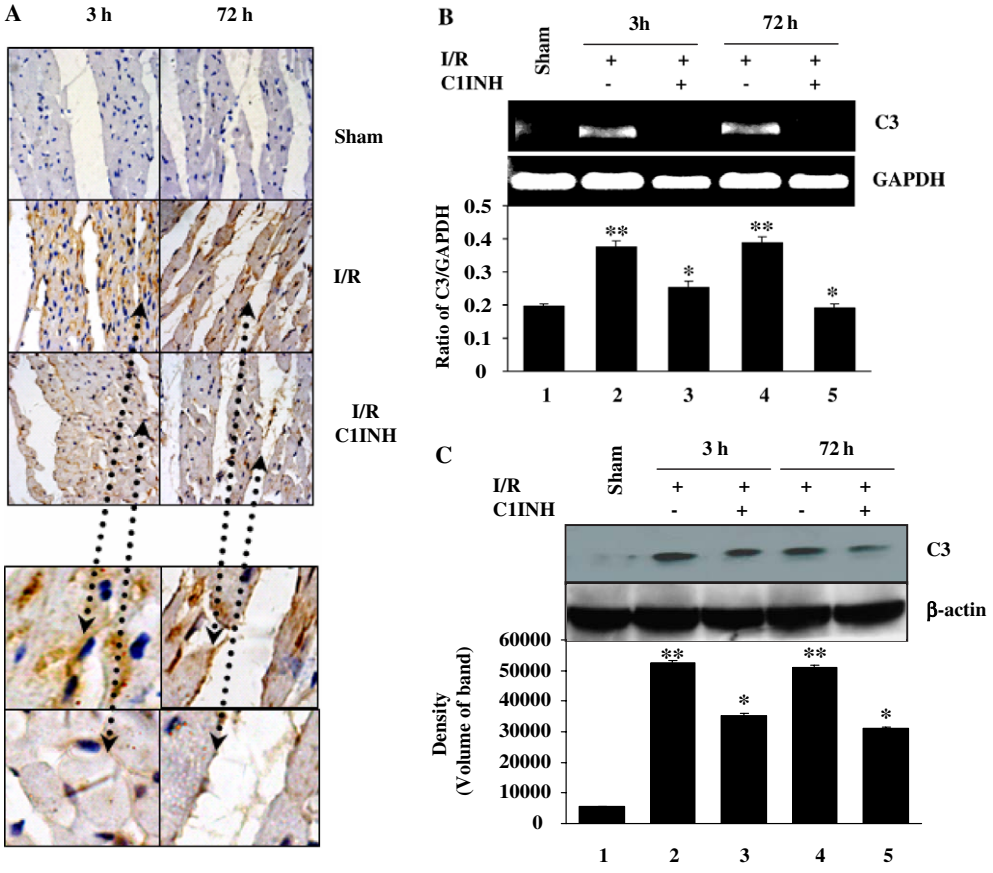

Fig. 1. Effect of C1INH on myocardial C3 staining and expression in myocardium in rat AMI. (A) Sectioned myocardial tissue from free wall of the myocardial ischemic area of I/R rats either treated with 0.9% NaCl ($n = 5$) or with C1INH ($n = 5$) or sham control ($n = 5$) was analyzed by immunohistochemical staining with anti-C3 antibody. Color photomicrographs of immunolocation of C3 were determined. Brown reaction product is present at sites of C3 proteins. (B) RNA was isolated from myocardial infarct tissue of either I/R rats receiving 0.9% NaCl ($n = 5$) or I/R rats treated with C1INH ($n = 5$) or sham control rats ($n = 5$). C3 mRNA expression was analyzed by RT-PCR. GAPDH was the internal control. Representative RT-PCR is shown for five experiments ($n = 5$). Ratio of the relative density of C3 mRNA in myocardial tissue after I/R. (C) Protein was isolated from myocardial infarct tissue of either I/R rats receiving 0.9% NaCl ($n = 5$) or I/R rats treated with C1INH ($n = 5$) or sham control rats ($n = 5$). Levels of C3 protein were analyzed by Western blot. β-Actin was the internal control. Representative Western blot is shown from five experiments ($n = 5$). Density of C3 protein (volume of the band) in myocardium after I/R for Western blot. $^{*}P < 0.05$ compared with C1INH-untreated I/R. $^{**}P < 0.05$ compared with sham control.

protein synthesis in cultured H9c2 rat heart myocytes induced by hypoxia/reoxygenation. Northern blot showed both hypoxia (24 h) alone and with subsequent reoxygenation (2 h) markedly enhanced C3 mRNA expression in heart myocytes (Fig. 2A). To determine whether mRNA upregulation by hypoxia/reoxygenation required new mRNA synthesis, the cells were treated with actinomycin D, an inhibitor of transcription, under condition of hypoxia/reoxygenation. Actinomycin D strongly prevented the increase of C3 mRNA during reoxygenation after hypoxia (Fig. 2B), suggesting that C3 expression is regulated at least the transcriptional level in response to hypoxia/reoxygenation. Similarly, changes of cytoplasmic C3 protein were observed by Western blot (Fig. 2C). A single dose of C1INH (150 μg/ml) blocked the increase of C3 mRNA induced by hypoxia (24 h) ($P < 0.05$) and reoxygenation (2 h) after hypoxia (24 h) ($P < 0.01$) (Fig. 2D). Furthermore, hypoxia/reoxygenation-induced levels of C3 protein could be decreased by C1INH (Fig. 2E).

C1INH interferes with hypoxia/reoxygenation-induced C3 deposition on, and binding to, the surface of rat heart myocytes

It has been reported that hypoxia/reoxygenation-induced vascular endothelial cells significantly increase iC3b deposition on endothelial cells and C3 deposition after hypoxia/reoxygenation is largely mediated by the classic complement pathway [12–14]. In H9c2 rat myocytes, a significant increase in C3 deposition was observed in reoxygenation (2 h) after hypoxia (24 h) compared to normoxic control ($P < 0.01$), whereas C1INH (150 μg/ml) treatment attenuated C3 deposition on the surface of myocardial cells ($P < 0.01$) (Fig. 3A). In addition, both C1INH and C3 had the ability to directly

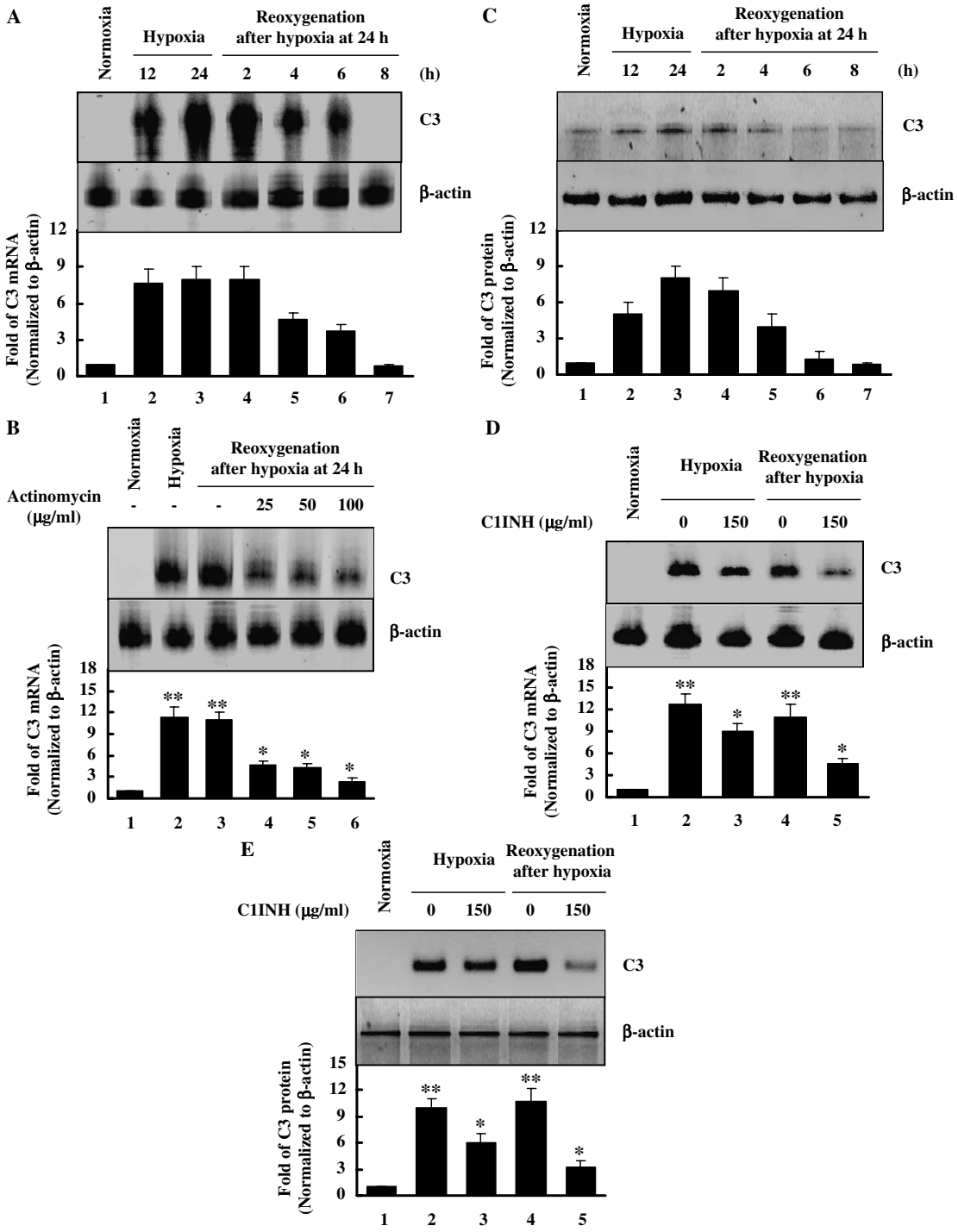

Fig. 2. Effect of C1INH on hypoxia/reoxygenation induces C3 mRNA expression and protein synthesis in rat H9c2 heart myocytes. (A) RNA was isolated from rat H9c2 heart myocytes with either hypoxia alone or hypoxia/reoxygenation. C3 mRNA expression was analyzed by Northern blot. β-Actin was the internal control. Representative Northern blot is shown from three experiments. (B) Rat myocardial cells were exposed to hypoxia alone or reoxygenation for 2 h after hypoxia for 24 h with treatment of actinomycin D for 2 h, and Northern blot was conducted. $^{*}P < 0.05$ compared with C1INH-untreated I/R. $^{**}P < 0.05$ compared with sham control. (C) Cytoplasmic extracts were isolated from rat H9c2 heart myocytes with hypoxia/reoxygenation. C3 protein was analyzed by Western blot. β-Actin was the internal control. Representative Western blot is shown for three experiments. Quantitative graph from triplicate experiments is shown. All values are means ± SEM for three experiments. (D) RNA was isolated from rat H9c2 heart myocytes with either hypoxia alone or hypoxia/reoxygenation in the presence or absence of C1INH. C3 mRNA expression was analyzed by Northern blot. β-Actin was the internal control. Representative Northern blot is shown from three experiments. (E) Cytoplasmic extracts were isolated from rat H9c2 heart myocytes with hypoxia/reoxygenation. C3 protein was analyzed by Western blot. β-Actin was the internal control. $^{*}P < 0.05$ compared with C1INH-untreated I/R. $^{**}P < 0.05$ compared with control.

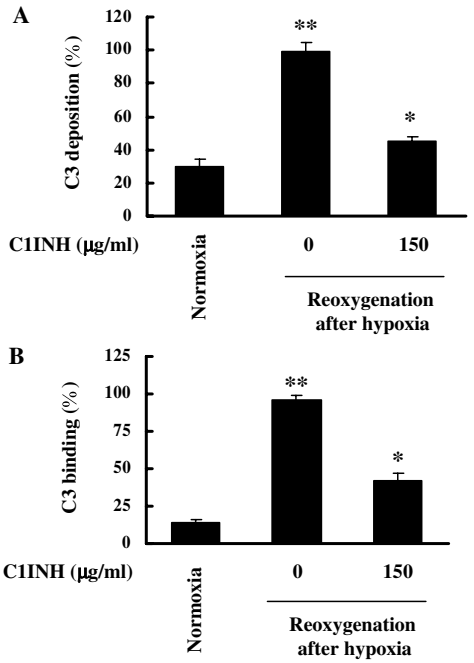

Fig. 3. Effect of C1INH on hypoxia/reoxygenation-induced C3 deposition on, and binding to, the surface of rat H9c2 heart myocytes (A) Hypoxia/reoxygenation-induced C3 deposition from rat plasma on the surface of rat H9c2 heart myocytes in the presence or absence of C1INH (150 μg/ml) was analyzed by ELISA. Representative result is shown for three experiments. $^{*}P < 0.05$ compared with hypoxia/reoxygenation. All values are means ± SEM for three experiments. (B) Hypoxia/reoxygenation-induced C3 binding to the surface of rat H9c2 heart myocytes in the presence or absence of C1INH (150 μg/ml) was analyzed by ELISA. $^{*}P < 0.05$ compared with hypoxia/reoxygenation group. $^{**}P < 0.05$ compared with control.

bind to cultured rat myocardial cells. C1INH (150 μg/ml) significantly reduced the binding of C3 to heart myocytes ($P < 0.01$) (Fig. 3B).

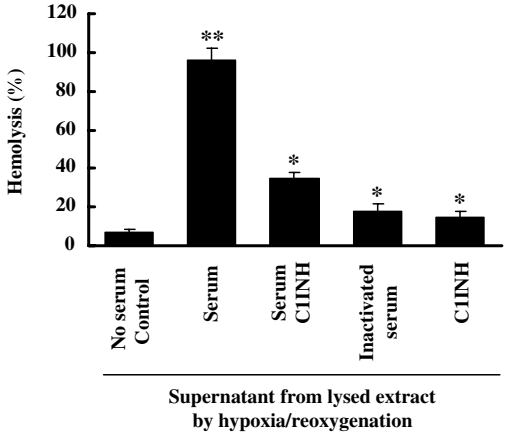

Fig. 4. Effect of C1INH on C3-mediated sensitized erythrocyte hemolysis. Sensitized chicken erythrocytes incubated with C3-deficient serum and the supernatant of myocardial cells treated with C1INH were analyzed for hemolysis assay. $^{*}P < 0.05$ compared with C1INH-untreated I/R. $^{**}P < 0.05$ compared with control.

C1INH inhibits hypoxia/reoxygenation-induced myocardial C3 activity

To further investigate myocardial C3-mediated complement activation, the sensitized erythrocytes were incubated with C3-deficient sera and hypoxia/reoxygenation-induced myocardial supernatant without treatment of C1INH. Hemolysis was increased to 96 ± 5.9% ($P < 0.01$) compared with the no serum control. The percentage of hemolysis of the sensitized erythrocytes was significantly reduced to 35 ± 3.5% ($P < 0.01$) when these cells were co-incubated with C3-deficient sera and C1INH-treated myocardial supernatant (Fig. 4).

Discussion

The previous data demonstrated the cardioprotective effects of C1INH in myocardial I/R injury via inhibition of complement system, neutrophil infiltration, and endothelial activation. We further demonstrated that C1INH directly suppresses local myocardial C3 expression in ischemic myocardial area of rat AMI. *In vitro*, hypoxia/reoxygenation-induced C3 mRNA expression in rat myocardial cells is downregulated by treatment with C1INH. Both C3 and C1INH bind to the surface of rat myocardial cells induced by hypoxia/reoxygenation. However, C3 fails to bind to the C1INH bound rat heart myocytes. Finally, C1INH has the ability to reduce lysis of the complement sensitive erythrocytes via inhibiting C3 activity by analysis of hemolysis. Therefore, C1INH has an anti-ischemia/reoxygenation in myocardial cell injury via regulation of local myocardial C3 activity. C1INH has been proved to reduce infarct size, reduce neutrophil accumulation in myocardium, reduce plasma level of creatine kinase, and inhibit the activation of C1 [11,15–17]. The mechanisms of cardioprotective effect appear to be due to blocking of complement activation and reduced endothelial adhesion molecule expression with subsequent reduced polymorphonuclear leukocyte–endothelium interaction, resulting in diminished cardiac necrosis [18]. Similar beneficial effects have been observed in other organ reperfusion injury [19–21]. Recently, the systemic administration of C3-specific siRNA is capable of entering renal cell and reducing renal C3 synthesis [22]. Collectively, complement system activation and neutrophil infiltration play an important role in I/R injury and tissue salvage may be achieved by inhibition of complement pathway. Localization of complement components (i.e., C1q, C3, C4, and C5) is involved in infarct myocardium [23–28]. The activation of complement after I/R injury has been attributed to different pathways [8,27,29,30]. Liver produces the highest C3 level under normal conditions, but heart tissue undergoing I/R produces substantially higher level of C3 component [31]. Local C3 synthesis may contribute to complement-dependent processes, including the mediation of I/R injury [32] and immune-mediated tissue injury [33]. Many ways have been studied: blocking key proteins of the cascade reaction; preventing C3 cleavage [18].

Studies have demonstrated that increases in circulating C3a were attenuated by C1INH treatment [9]. It is likely that C1INH contributes to protection from myocardial cell injury via both inhibition of systemic complement activation such as C5a and C5b-9, and downregulation of proinflammatory events including cytokine release, adhesion molecule expression, and neutrophil accumulation [27,28]. We further demonstrate that local production and activation of complement proteins may contribute significantly to the degree of ischemic injury to the myocardium and local complement expression is augmented by reperfusion. Therefore, a target for suppression of local complement compound(s) is a useful therapeutic approach to some patients with AMI in clinical setting.

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