In Vitro Effect of Elastase and Cathepsin G from Human Neutrophils on Creatine Kinase and Lactate Dehydrogenase Isoenzymes

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The polymorphonuclear granulocyte, or neutrophil, has been implicated as a mediator of tissue-destructive events because it releases the preformed proteolytic enzymes elastase and cathepsin G, and, as a result of myeloperoxidase action, hypochlorous acid. We show that elastase inactivates and fragments creatine kinase isoenzymes CK-2 and CK-3, and, to a lesser extent, lactate dehydrogenase (LD) isoenzyme LD-1, whereas cathepsin G acts only on CK-2. Both neutrophil enzymes act on LD-3. The course of inactivation was followed by measuring the loss of catalytic activity at 37°C. The evidence for fragmentation was obtained by gel filtration; electrophoresis after sample treatment with sodium dodecyl sulfate and 2-mercaptoethanol was less satisfactory for this purpose. Hypochlorous acid inactivates CK activity by about 75% at concentrations as low as 8 μmol/L and totally at concentrations >140 μmol/L, whereas LD activity is not affected until concentrations exceed 200 μmol/L. After a myocardial infarction, the number of neutrophils increases; they are triggered and concentrate around damaged myocardial tissue. Our data suggest that neutrophils may inactivate and fragment "cardiac" enzymes released from such damaged tissue.

Indexing Terms: polymorphonuclear granulocyte · myocardial infarction

The polymorphonuclear granulocyte, or neutrophil, has been implicated as a mediator of tissue-destructive events (1). The neutrophil can synthesize and release a host of toxic agents produced either from the plasma membrane or from one of the intracellular azurophil or specific granules (2). The azurophil granules contain a variety of proteins, including elastase (lysosomal or neutrophil-derived; EC 3.4.21.37) and cathepsin G (EC 3.4.21.20). [Neutrophil elastase differs from pancreatic elastase (EC 3.4.21.36) in its specificity for synthetic substrates and its sensitivity to inhibitors.] Both of these neutrophil enzymes are neutral serine proteinases and hydrolyze a wide range of proteins (3). For example, elastase hydrolyzes type VIII collagen (4), α2-plasmin inhibitor (5), C1 inactivator (5), membrane-bound C3bi (6), fibronectin (7), factor IX (8), and fibrinogen (9), and has been implicated in the conversion of big endothelin 1 to endothelin 1 (10). Both elastase and cathepsin G participate in the hydrolysis of basement membrane laminin (11) and subendothelial matrix (12, 13); cathepsin G has been implicated in the removal of the calcium-binding domain of factors II (14), VII (15), IX (15), X (16), and protein C (17).

Activation of the neutrophil after a myocardial infarction (MI) results in a complex series of reactions, including the release of enzymes into the surrounding environment (18). A neutrophil elastase–α1-proteinase inhibitor complex has been detected in MI patients, particularly after successful thrombolyis (19). Concentrations of a plasma elastase-derived fibrinopeptide (Bβ 30-43) have been reported as increasing fivefold in patients after an MI (20, 21), and this finding has been suggested as further evidence of infarction-induced neutrophil activation.

Early studies in myocardial infarct sizing seemed to suggest that not all the creatine kinase (EC 2.7.3.2; CK) or lactate dehydrogenase (EC 1.1.1.27; LD) released from the myocardium could be subsequently recovered in serum (22–24). Hermens and Willems (25) argued that these conclusions were based on different methods of data analysis and that there is now much evidence to suggest that the cytosolic isoenzymes, such as CK-3, CK-2, and LD-1, are transported rapidly and quantitatively to the blood serum. Nonetheless, these authors used equation constants obtained from the disappearance of infused enzymes from serum under presumably stable physiological conditions. However, a patient with a developing MI is not stable; as suggested earlier, there is a marked granulocytosis, and neutrophils are activated. Consequently, we wished to establish whether the neutrophil proteinases elastase and cathepsin G participate in the destruction of "cardiac" isoenzymes CK-2, CK-3, and LD-1 in serum. Our findings suggest that both proteinases have the ability to inactivate and fragment these isoenzymes, as indicated in a preliminary report (26).

Materials and Methods

Chemicals

Human LD-1, LD-2, LD-3, and LD-5 were either prepared in our laboratory (27, 28) or purchased from Sigma Chemical Co., St. Louis, MO. Human CK-2 and CK-3 were purchased from Scripps Laboratories, San Diego, CA. Human leukocyte elastase and cathepsin G were purchased from Biodesign International, Kennebunkport, ME. α1-Proteinase inhibitor (α1-antitrypsin) and α2-macroglobulin were purchased from Sigma. All other chemicals were obtained either from Sigma or from BDH Chemicals Canada Ltd., Toronto, ON.

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Methods

Assay of CK and LD. The assays of CK and LD were performed as previously described (29, 30).

Assay of elastase and cathepsin G. We used N-methoxy succinyl-ala-lyl-prol-val-2-nitroanilide, at a final concentration of 1 mmol/L, as substrate for the assay of elastase activity and succinyl-ala-lyl-prol-phenylalanyl-2-nitroanilide, at a final concentration of 4 mmol/L, as substrate for the assay of cathepsin G activity (31); we dissolved both substrates in dimethyl sulfoxide before addition to the assay system. The assay buffer was 90 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt (HEPES), pH 7.50, containing 450 mmol/L NaCl and 1.28 mol/L dimethyl sulfoxide. We used an absorbivity (L·mol⁻¹·cm⁻¹) of 8800 at 410 nm and an assay temperature of 37 °C.

Ultrafiltration. We used a 3-mL ultrafiltration cell with a YM 10 (10,000 M₀ cutoff) membrane (Amicon, Lexington, MA).

Incubation experiments. Enzyme activity was preserved by the addition of bovine serum albumin (1 g/L) before ultrafiltration of the enzymes to remove stabilizers such as ammonium sulfate, glycerol, EDTA, and thiol agents. Albumin was omitted from experiments requiring electrophoresis or liquid chromatography.

Enzymes were suspended in 20 mmol/L Tris buffer, pH 7.4. The initial activities of the elastase and cathepsin G solutions, dissolved in the Tris buffer, were 3.5 kU/L (100 mg/L, or about 3.4 μmol/L) and 3.7 kU/L (330 mg/L, or about 14 μmol/L), respectively. These solutions were diluted with Tris buffer to produce working solutions with final assay concentrations of 100, 200, and 300 mmol/L when 50 μL was used in a total incubation volume of 350 μL. Incubation temperature was 37 °C, and 50-μL volumes were periodically withdrawn for the assay of residual enzyme activity. Control tubes contained only the enzyme suspended in the Tris buffer. For subsequent electrophoresis or chromatographic separations, aliquots of 100 or 200 μL were withdrawn, respectively.

Hypochlorous acid was obtained immediately before use by generation from NaOCl as described by Wasil et al. (32). We used an absorptivity (L·mol⁻¹·cm⁻¹) of 100 at 235 nm (33) at room temperature. In incubation experiments with HOCl, we used phosphate-buffered saline [per liter, 140 mmol of NaCl, 2.7 mmol of KCl, 16 mmol of Na₂HPO₄, and 2.9 mmol of KH₂PO₄, pH 7.4 (32)].

Fast Protein Liquid Chromatography (FPLC). We used the Automated FPLC® System and FPLCmanager® software (Pharmacia LKB Biotechnology, Uppsalā, Sweden) with a Superose gel filtration HR 12 column (10 × 300 mm; flow rate, 18 mL/h). The sample size was 200 μL. All reagents and samples were filtered through 0.22-μm (pore size) Millipore membranes or Ultrafree-MC filter units before application to the column. Column eluates were monitored at 280 nm and collected in 500-μL fractions when required.

Electrophoresis. We used the PhastSystem® (Pharma-
cia). All separations were performed on 20% high-density polyacrylamide gels in the presence of sodium dodecyl sulfate and 2-mercaptoethanol according to the PhastSystem protocol. We examined these gels for peptide fragments by using the PhastTransfer® electrophoretic transfer system onto Millipore Immobilon PVDF or Pharmacia nitrocellulose transfer membranes. We stained the polyacrylamide gels, using the PhastSystem combined Coomassie R 350 and silver stain procedure, and the PVDF or nitrocellulose membranes by the Kovarik silver stain procedure (34).

Results and Discussion

In MI the white blood cell count increases within 2 h of onset and can rise to about 20 × 10⁶ cells/L (reference range <11 × 10⁶ cells/L) and neutrophils may constitute as much as 90% of the white cell population (35). When a tissue is injured, neutrophil chemotactic factors are released. These factors diffuse locally and adhere to the neutrophil's cell surface receptors, where they promote adherence and aggregation of the neutrophil to the endothelial cells of capillaries and venules near the site of injury; the neutrophils then move through the endothelial cell junctions towards the source of the factors (36-38). Subsequently, the neutrophils adhere to the damaged cells. The preformed granules (azurophil, specific, and other types) are secreted (39) and there is a marked increase in oxygen consumption (the respiratory burst). The azurophil granules contain a large family of enzymes including elastase and cathepsin G. These cause tissue damage, and we have shown that both these proteinases inactivate and fragment the LD and CK isoenzymes.

In this study we had to consider the likely concentration of these proteinases. The neutrophil count is not necessarily a reliable measure, for three reasons. First, the majority of neutrophils do not circulate as they sequester in the postcapillary venules (40); second, neutrophils move freely from the intravascular to the extracellular space in response to a chemotactic factor (40); and third, proteinase release is dependent on neutrophil activation (37). Neutrophil proteinases do not exist free in blood; they complex with α₁-proteinase inhibitor (elastase) or α₁-antichymotrypsin (cathepsin G) (41). The elastase-α₁-proteinase inhibitor complex has been measured in blood in patients with stable angina after percutaneous transluminal coronary angioplasty (42) or following thrombolysis treatment after MI (19, 21, 43). Amounts up to 20 nmol/L were detected. Because elastase is released after neutrophil diapedesis, we can estimate an upper limit of 300 nmol throughout the extracellular fluid, which has a volume of about 15 L (44). Given that neutrophils concentrate at the site of tissue injury, it is reasonable to assume that the elastase concentration might be on the order of 300 nmol/L at the myocardial surface. Accordingly, we used concentrations of 100–300 nmol/L in our experiments.

Effect of neutrophil elastase and cathepsin G on CK isoenzyme activities. Preliminary 3-h incubations were performed with elastase and cathepsin G at final con-
centrations of 100, 200, and 300 nmol/L. CK-2 and CK-3 activity is quickly lost after the addition of elastase (Figure 1A). Increasing the elastase concentration suggests that the effect at 300 nmol/L is close to a plateau (and our experience using higher concentrations up to 600 nmol/L confirms that suggestion). In the case of cathepsin G (Figure 1B), however, CK-2 is inactivated much less than by elastase, and CK-3 is hardly inactivated. The incremental steps suggest that the addition of more cathepsin G would further inactivate both isoenzymes; we have confirmed this suggestion by using concentrations up to 500 nmol/L. Timed studies, with neutrophil enzymes at concentrations of 300 nmol/L (Figure 2), show a very rapid inactivation of both CK-2 and CK-3 by elastase, but also a much longer time required for cathepsin G action on CK-3 under the chosen experimental conditions.

**Effect of neutrophil elastase and cathepsin G on the CK isoenzyme gel filtration patterns.** Before experiments with any of the isoenzymes, the gel filtration patterns of unincubated and postincubation elastase and cathepsin G, at the activities used in the experiments, were established. Neither coeluted with the CK isoenzymes or contributed significantly to their fragmentation patterns (data not shown). The gel filtration patterns (Figure 3) clearly show fragmentation of both CK isoenzymes and a reduction in the amount of the intact molecules, although the effect is much less in the case of CK-3. This confirms that the mechanism of inactivation involves proteolysis, although it should be noted that CK-3 fragmentation by cathepsin G (Figure 3B) is not accompanied by enzyme inactivation (Figure 2B).

Electrophoresis studies were performed in parallel with the gel filtration separations. The results were much less definite than those of gel filtration, and have therefore been omitted.

**Effect of neutrophil elastase and cathepsin G on LD isoenzyme activities.** Preliminary 3-h incubations were performed with elastase and cathepsin G at final concentrations of 100, 200, and 300 nmol/L. LD-1 is inactivated by the action of elastase but cathepsin G has no effect (Figure 4). We also found that increasing the concentration of either neutrophil enzyme increased both the rate and extent of enzyme inactivation. The unexpected finding was the quite marked inactivation of LD-3 by both proteinases. In contrast, both LD-2 and LD-5 are little affected by either proteinase. It is therefore difficult to draw firm conclusions about the effect of an increased M-subunit composition on LD isoenzyme proteinolysis. Timed studies (Figure 5), done at concen-

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Fig. 1. Effect of various concentrations of human neutrophil elastase (A) and cathepsin G (B) on human CK-2 and CK-3 inactivation

![Graph A](image)

![Graph B](image)

Fig. 2. Effect of human neutrophil elastase and cathepsin G on human CK-2 and CK-3 activities

Elastase and cathepsin G were each 300 nmol/L. Each plot shows a control incubation (C), and the effect of the addition of elastase (E) or cathepsin G (O). The 95% confidence intervals are shown where the interval is large enough to plot.
Although concentrations of 300 nmol/L, show the effect of elastase to be LD-3 > LD-1 > LD-2 > LD-5, whereas the effect of cathepsin C is LD-3 > LD-2 > LD-1 > LD-5.

Effect of neutrophil elastase and cathepsin G on the LD isoenzyme gel filtration patterns. The gel filtration patterns of elastase and cathepsin G were established; they did not coelute with the LD isoenzymes or contribute significantly to their fragmentation patterns (data not shown). Elastase certainly produces fragmentation, and reduction in isoenzyme concentration in some cases, but the effect of cathepsin C is less marked (Figure 6). Although LD-5 shows a fragmentation pattern, the loss of LD activity was insignificant. Presumably, the proteolysis that did occur did not affect the functioning of the active site.

However, one can reasonably ask if the presence of serum, or serum components, would prevent such proteolysis. Proteinase inhibitors in blood serum are the third largest group of functional proteins after albumin and the immunoglobulins, and they constitute nearly 10% of the total protein content (41). The serine proteinase inhibitors have been given the generic name serpins (45), and serpins are now known to have an important control function for proteolysis in thrombosis, shock, and inflammation. The three serpins of interest, when considering neutrophil neutral serine proteinases, are α1-proteinase inhibitor (α1-antitrypsin), α2-macroglobulin, and α1-antichymotrypsin. The former is regarded as the specific serum inhibitor of neutrophil elastase; the latter is the specific inhibitor of cathepsin G (42). α2-Macroglobulin not only inhibits serine proteinases but also inhibits the metalloproteinases. In addition to these serpins, a fourth inhibitor, secretory leukocyte protease inhibitor (46), is found in high concentrations in mucus fluids. In the present work the inhibitory effect of α1-proteinase inhibitor (α1-antitrypsin) and α2-macroglobulin on neutrophil elastase and cathepsin G was confirmed by using LD-2 as the proteinase "substrate" (data not shown). This anti-proteinase inhibition was abolished, in the case of α1-proteinase inhibitor, after its oxidation with HOCl (32) (Table 1). In the presence of the phosphate buffer, elastase and cathepsin G proteolysis is markedly slower than that in Tris buffer (Figure 5B). HOCl had no effect, as such, on LD activity at the concentrations used (about 20 μmol/L). However, when the HOCl concentration was >200 μmol/L, endogenous LD-2 activity was reduced to zero. No HOCl experiments were performed with α2-macroglobulin.

Effect of HOCl on CK isoenzymes. Another product of the respiratory burst that can cause tissue damage is hypochlorous acid. Weiss estimates that from 2500 to 5000 nmol of HOCl may be generated by 25 × 10⁶ neutrophils and that 10⁶ maximally triggered neutrophils may produce 200 nmol of HOCl in a 2-h period (1). HOCl is an extremely reactive species that can oxidize a wide variety of biological molecules. CK-3 was exposed to a range of HOCl concentrations (5 to 800 μmol/L) in

Fig. 3. Gel filtration patterns of CK-2 (A) and CK-3 (B) after incubation with human neutrophil elastase and cathepsin G. The control patterns were obtained without prior incubation, and CK activity was detected only in association with the peak eluting at about 11 mL.

Fig. 4. Effect of various concentrations of human neutrophil elastase (A) and cathepsin G (B) on human LD-1, LD-2, LD-3, and LD-5 inactivation.
the presence of bovine serum albumin (1 g/L). Endogenous CK activity was completely lost with HOCl concentrations above 140 μmol/L, but below that, CK activity was inhibited by about 75% (data not shown). The CK assay mixture was less sensitive than CK itself to the effects of HOCl, so the inhibitory effect of HOCl is entirely due to its direct action, at low concentrations, on the active site of CK. The active site of CK contains a thiol group of cysteine essential for its catalytic activity; thus, iodoacetate alkylation of this thiol group leads to an irreversible loss of activity (47). HOCl preferentially oxidizes thiol groups (33, 48) before reacting with other chemical species. These findings explain our observation of the inactivating effect of HOCl on CK-3 activity.

Effect of HOCl on α1-proteinase inhibitor. Table 1 shows that the presence of HOCl inhibits the action of α1-proteinase inhibitor on both elastase and cathepsin G. These findings are explicable in terms of the HOCl oxidation of the methionine residue at position 358 of the α1-proteinase inhibitor to methionine sulfoxide (41). Oxidation of the α1-proteinase inhibitor causes a 2000-fold decrease in the rate of association between it

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Table 1. Effect of HOCl on α1-Proteinase Inhibitor
Reactions with Neutrophil Proteinases Determined by Using LD-2 Activity as an Indicator of Proteolysis

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD-2</td>
<td>34.8</td>
<td>29.9</td>
</tr>
<tr>
<td>LD-2 + α1-proteinase inhibitorb</td>
<td>33.7</td>
<td>30.7</td>
</tr>
<tr>
<td>LD-2 + HOClc</td>
<td>29.8</td>
<td>28.6</td>
</tr>
<tr>
<td>LD-2 + elastased</td>
<td>35.7</td>
<td>12.5</td>
</tr>
<tr>
<td>LD-2 + elastased + α1-proteinase inhibitorc</td>
<td>34.9</td>
<td>33.8</td>
</tr>
<tr>
<td>LD-2 + elastased + α1-proteinase inhibitorc + HOClf</td>
<td>35.4</td>
<td>14.9</td>
</tr>
<tr>
<td>LD-2 + cathepsin Gg</td>
<td>35.7</td>
<td>12.1</td>
</tr>
<tr>
<td>LD-2 + cathepsin Gg + α1-proteinase inhibitorh</td>
<td>28.8</td>
<td>28.8</td>
</tr>
<tr>
<td>LD-2 + cathepsin Gg + α1-proteinase inhibitorh + HOClf</td>
<td>39.4</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* All LD-2 preparations contained bovine serum albumin (1 g/L). Reaction mixtures were incubated for 20 h at 37 °C.
* 2.4 μmol/L
* 20 μmol/L
* 800 nmol/L
* 1.2 μmol/L
and neutrophil elastase, resulting in an in vivo increase of elastase half-life from 0.6 ms to 1.2 s. Thus, oxidized \( \alpha_1 \)-proteinase inhibitor is ineffective in preventing proteolysis of susceptible proteins. Similar evidence has been presented for the oxidative inactivation of both \( \alpha_2 \)-macroglobulin (1) and secretory leukocyte protease inhibitor (49).

In conclusion, the two neutrophil components that cause tissue destruction—secreted proteolytic enzymes and HOCl—can be considered in terms of a single, integrated mechanism (1). The generated HOCl creates an oxidizing screen around the neutrophil. Antiproteinases within this screen are oxidized and rendered less effective as inhibitors of the secreted neutrophil elastase. Accordingly, elastase attacks susceptible proteins within this oxidizing screen. In addition, HOCl may oxidize other proteins, such as CK, and render them enzymatically inactive. Also, the neutrophil metalloproteinases, collagenase (EC 3.4.24.7) and gelatinase (EC 3.4.24.24 or 35), are activated by HOCl action and have been implicated in the proteolysis of \( \alpha_1 \)-proteinase inhibitor (1). A similar effect has been ascribed to a third neutrophil metalloproteinase (50). Also, the specific inhibitor of cathepsin G, \( \alpha_1 \)-antichymotrypsin, can be cleaved by uninhibited neutrophil elastase action (51), thus reducing inhibition of cathepsin G within the oxidative screen. Our in vitro data are therefore consistent with these mechanisms. Inactivation and fragmentation of the "cardiac" enzymes can evidently occur, although we accept that such a conclusion remains to be confirmed in vivo.

References