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Angiotensin II Generation at the Cell Surface of Activated Neutrophils: Novel Cathepsin G-Mediated Catalytic Activity That Is Resistant to Inhibition[1]

Caroline A. Owen[2] and Edward J. Campbell

Human neutrophils express inducible, catalytically active cathepsin G on their cell surface. Herein, we report that membrane-bound cathepsin G on intact neutrophils has potent angiotensin II-generating activity. Membrane-bound cathepsin G on activated neutrophils 1) converts both human angiotensin I and angiotensinogen to angiotensin II; 2) expresses angiotensin II-generating activity equivalent to 8.6 ± 2.3 (±SD) × 10^{-18} mol of free cathepsin G (5.2 ± 1.4 × 10^6 molecules)/cell; and 3) has similar high affinity for angiotensin I compared with free cathepsin G (K_m = 5.9 × 10^{-4} and 4.6 × 10^{-4} M; k_{cat} = 4.0 and 2.0/s, respectively). In marked contrast to soluble cathepsin G, membrane-bound enzyme was substantially resistant to inhibition by plasma proteinase inhibitors and converted angiotensin I to angiotensin II even in undiluted plasma. There was a striking inverse relationship between inhibitor size and its effectiveness against membrane-bound cathepsin G activity. α_1-Antichymotrypsin was a markedly ineffective inhibitor of membrane-bound enzyme (IC_{50} = 2.18 μM and 1.38 nM when tested against 1 nM membrane-bound and free cathepsin G, respectively). These data indicate that membrane-bound cathepsin G expressed on neutrophils is an inducible and mobile angiotensin II-generating system that may exert potent local vasoactive and chemoattractant properties at sites of inflammation. ***The Journal of Immunology*****, 1998, 160: 1436–1443.**

Angiotensin II (ANGII)[3] is an octapeptide that has potent vasoconstrictor and aldosterone-secreting activities and thereby plays a central role in the regulation of blood pressure and fluid homeostasis in man (1). ANGII can also modulate inflammatory and immune processes, since its biologic activities include 1) stimulation of smooth muscle contraction (2), 2) enhancement of vascular permeability (3), 3) induction of PG synthesis by endothelial cells (2), 4) suppression of lymphocyte proliferation and differentiation (4), and 5) promotion of the accumulation of polymorphonuclear neutrophils (PMN) and mononuclear cells in vitro (5–10).

In the conventional initial step for the generation of systemic ANGII, renin (EC 3.4.99.19; a renal acid proteinase) cleaves angiotensin I (ANGI; a biologically inactive decapeptide) from the 50-kDa plasma glycoprotein angiotensinogen. ANGI is then converted to ANGII by dipeptidyl carboxypeptidase (kinase II, EC 3.4.15.1), which is expressed by endothelial cells of most tissues (11). Recently, a major alternative pathway has been described for the local generation of ANGII within the human heart and blood vessels; human heart chymase, a serine proteinase that is expressed by cardiac mast cells, mesenchymal interstitial cells and endothelial cells has very high substrate specificity for ANGI and can rapidly convert it to ANGII (12–14). Several other enzymes, including cathepsin G, kallikrein, chymotrypsin, and tonin, have been shown to produce ANGII from ANGI in vitro (12, 15), but the physiologic significance of these latter ANGII-generating activities in vivo has not been elucidated.

Cathepsin G (EC 3.4.21.20) is a serine proteinase that is present in high concentrations in the azurophil granules of human PMN and peroxidase-positive granules of proinflammatory monocytes (16–19). In contrast to human leukocyte elastase, it is rather weakly proteolytic, and its natural substrate(s) and biologic activities are poorly understood. Several potentially important activities of cathepsin G have been described based upon in vitro activities of the purified enzyme while in solution. In particular, purified cathepsin G has been shown in vitro to rapidly convert ANGI to ANGII and can also generate ANGII directly (albeit more slowly) from angiotensinogen (20–22). Conversion of ANGI to ANGII by purified cathepsin G in vitro occurs at rates sufficiently rapid to be of biologic significance and is one of the fastest reaction rates known for cathepsin G (K_m = 2.2 × 10^{-4} M; k_{cat} = 3.4/s) (22). However, the relevance of this observation to the in vivo activity of the enzyme has been difficult to determine, since 1) cathepsin G is poorly soluble in isotonic solutions (23); 2) little cathepsin G is released into extracellular fluid when azurophil granule contents are released from PMN in response to physiologic stimuli (24, 25); and 3) high affinity inhibitors of cathepsin G, such as α_1-antichymotrypsin and α_2m, are present in the extracellular space. In this respect, it is noteworthy that we and others have recently shown that cathepsin G is expressed on the surface of human PMN (25–27). Cell surface expression of cathepsin G on PMN is strikingly

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[3] Abbreviations used in this paper: ANGII, angiotensin II; PMN, polymorphonuclear neutrophils; ANGI, angiotensin I; SAAPF-pNA, succinyl-Ala-Ala-Phe-*p*-nitroanalide; α_1-Ach, α_1-antichymotrypsin; SAAF-AFC, succinyl-Ala-Ala-Phe-7-amino-4-trifluoromethyl-coumarin; MSAPV-AFC, methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethylcoumarin; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine); SLPI, secretory leukoprotease inhibitor; HLE/CMK, methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone; CG/CMK, *Z*-Gly-Leu-Phe chloromethylketone; V_{max}, maximum velocity.

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up-regulated by signals that are relevant to infection and inflammation, and membrane-bound enzyme is catalytically active against a synthetic oligopeptide substrate (25, 26).

We now report that cathepsin G, when bound to the cell surface of activated PMN, has potent ANGII-generating activity. Moreover, in marked contrast to freely released cathepsin G, membrane-bound enzyme is substantially resistant to inhibition by naturally occurring proteinase inhibitors. These data indicate that persistently active cathepsin G on the cell surface of activated PMN provides a mechanism by which these cells can locally generate ANGII at sites of their accumulation, and thereby modulate blood flow and cellular accumulation during the inflammatory response even in the presence of proteinase inhibitors present in the local microenvironment.

Materials and Methods

Reagents

Human ANGI, human ANGII, captopril, pepstatin A, FMLP, paraformaldehyde, glutaraldehyde, and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanalide (SAAPF-pNA) were purchased from Sigma Chemical Co. (St. Louis, MO). Succinyl-Ala-Ala-Phe-7-amino-4-trifluoromethylcoumarin (SAAF-AFC) and methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethylcoumarin (MSAPV-AFC) were purchased from Enzyme Systems Products (Dublin, CA). Human plasma angiotensinogen was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). 8-Hydroxyquinoline was obtained from New England Nuclear (Boston, MA). 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (platelet-activating factor (PAF)) was purchased from Bachem (Torrance, CA).

Serine proteinases and proteinase inhibitors

Human leukocyte elastase and cathepsin G were purified from purulent sputum (28). Proteinase 3 was a gift from Dr. J. R. Hoidal, M.D., University of Utah Health Sciences Center (Salt Lake City, UT). α_1-Proteinase inhibitor was obtained from Bayer Corp. (New Haven, CT). α_1-Antichymotrypsin and PMSF were purchased from Sigma Chemical Co. Eglin C was obtained from Dr. H. P. Schnebli, Ciba-Geigy Ltd. (Basel, Switzerland). Human recombinant secretory leukocyte proteinase inhibitor (SLPI) was provided by Amgen Boulder, Inc. (Boulder, CO). Methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone (HLE/CMK) and *Z*-Gly-Leu-Phe chloromethyl ketone (CG/CMK) were purchased from Enzyme Systems Products. The human leukocyte elastase preparation was 98% active when titrated against the active site titrant *Z*-Ala-Ala-Pro-Alanitrophenol (29). The activity of the α_1-proteinase inhibitor was measured using active-site-titrated human leukocyte elastase (30) and was 44% active. The activity of cathepsin G was measured using active-site-titrated α_1-proteinase inhibitor and was 100% active. Eglin C, SLPI, and α_1-antichymotrypsin were titrated against cathepsin G and were 100, 91, and 92% active, respectively. All concentrations of enzymes and proteinase inhibitors refer to the amount of active protein present.

Cell isolation and activation

Human PMN and PBMC were obtained from peripheral blood using Ficoll-Hypaque (31). Differential counting was performed on cytocentrifuge preparations of PMN and revealed that >95% of the cells were PMN. The remainder of the cells were eosinophils. PMN were resuspended at 3×10^6/ml in HBSS (pH 7.4) and primed for 1 min at 37°C with PAF (10^{-9} M), then stimulated for 30 min at 37°C with FMLP (10^{-8} M). We have shown that these concentrations of agonists and incubation times result in optimal expression of cell surface-bound immunoreactive cathepsin G (25). Cells were then washed once in HBSS, fixed for 5 min at 4°C in PBS containing 3% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde, pH 7.4 (to prevent leakage of endogenous serine proteinases), and washed in HBSS. PMN that were exposed to PAF and FMLP were >95% viable, as assessed by release of lactate dehydrogenase activity using a commercially available kit (Sigma Chemical Co.) (25).

Where specified, exogenous cathepsin G or human leukocyte elastase was bound to PBMC and unstimulated PMN (26). The PBMC or PMN were fixed, resuspended in HBSS at 10^7 cells/ml, then incubated at 4°C for 60 min with and without exogenous cathepsin G or human leukocyte elastase (40 pmol/10^6 cells). The cells were washed twice in HBSS to remove unbound proteinase and fixed again where specified.

ANGII-generating activity expressed by fixed PMN and free cathepsin G

Fixed unstimulated or activated PMN (10^7) or free cathepsin G (50 nM) were incubated in PBS for 2 h at 37°C with ANGI (100 nM) in the presence or the absence of CG/CMK (600 μM) in a total volume of 750 μl. PMSF (500 μM) was added to terminate the reaction, then disappearance of ANGI was quantified in triplicate in cell-free supernatant fluids using an [^{125}I]ANGI RIA (New England Nuclear). To confirm that activated PMN can generate ANGII from ANGI, the products formed were identified by reverse phase HPLC, as described below.

To test the ability of membrane-bound cathepsin G to convert angiotensinogen to ANGII, free cathepsin G (40 nM) or fixed activated PMN (10^7/ml) were incubated for 30 min at 37°C with purified human angiotensinogen (100 μM). ANGII was identified and quantified in cell-free supernatant fluids by HPLC.

Reverse phase HPLC of ANGII

Cleavage products from angiotensinogen and ANGI were identified by gradient elution, reverse phase HPLC (21), using a 4.6×250 mm Vydac Protein C4 (214 TP, 300 Å pore) column (The Separations Group, Hesperia, CA) and a Hewlett-Packard 1090 liquid chromatograph with a 1040A diode array detector and a 3392A integrator (Hewlett-Packard, Avondlae, PA). Briefly, solvent A was 50 mM phosphoric acid adjusted to pH 3.5 with triethylamine, and solvent B was 50% acetonitrile in solvent A, with a linear gradient from 10 to 75% B over 18 min from the start of the injection. The area under the peak corresponding to ANGII was determined by integration and referenced to standards of human ANGII.

ANGI-converting activity of various PMN-derived serine proteinases

Purified cathepsin G, human leukocyte elastase, or proteinase 3 (20 nM) or fixed activated PMN with 20 nM cathepsin G activity were incubated at 37°C in a final volume of 200 μl of HBSS containing 300 μM ANGI for times varying from 1 to 45 min. PMSF (500 μM) was added, and the products formed were identified and quantified by HPLC.

Quantification of enzyme activities

Catalytically active cathepsin G was quantified on fixed activated PMN, fixed PMN, or PBMC that bound exogenous cathepsin G using SAAF-AFC, a fluorogenic substrate that is specific for cathepsin G (25). To exclude the possibility that intracellular cathepsin G activity can leak from fixed PMN, fixed activated PMN (10^8/ml in HBSS) were incubated for 2 h at 37°C, then cathepsin G was quantified in cell-free supernatant fluids using SAAF-AFC. The assay was sensitive to the release of 12.5 ng of cathepsin G/10^6 cells.

Catalytically active cathepsin G was also quantified on PMN and PBMC using ANGI as the substrate. Varying amounts of free cathepsin G (5–80 nM) or fixed cells (2.5×10^6/ml) were incubated for 15 min at 37°C with ANGI (300 μM). PMSF (1 mM) was added to terminate the reaction, then ANGII was quantified in cell-free supernatant fluids by HPLC.

The quantity of exogenously added human leukocyte elastase activity that bound to PBMC was quantified using MSAPV-AFC, a fluorogenic substrate that is specific for human leukocyte elastase, as described previously (32). Dipeptidyl carboxypeptidase angiotensin-converting enzyme activity (EC 3.4.15.1) on fixed PMN was measured using a fluorometric assay as described previously (33).

To assess the effect of fixatives on the catalytic activity of membrane-bound cathepsin G, fixed unstimulated PMN were incubated with exogenous cathepsin G, as described previously. The cells were washed in HBSS to remove unbound proteinase, then aliquots of the cell suspension were either fixed (as described above) or kept in PBS alone (26). Both groups were washed in HBSS, then assayed in triplicate (2.5×10^6 cells/assay) using 200 μM SAAPF-pNA (25). Unfixed and fixed, exogenously added, membrane-bound enzyme had similar amounts of cathepsin G activity (961.5 ± 41.0 and 942.5 ± 45.4 ($\pm$SD) ng/10^6 cells, respectively). These data indicate that our fixation process has minimal effect on the catalytic activity of membrane-bound cathepsin G on PMN.

Kinetics of ANGII generation

To determine saturation kinetic constants, catalytically equivalent amounts (20 nM) of free and membrane-bound cathepsin G on activated PMN were incubated in a total volume of 200 μl of HBSS for 15 min at 37°C with varying quantities of ANGI (50–800 μM). The reaction was stopped by the addition of PMSF (500 μM), and cell-free supernatant fluids were assayed in triplicate for ANGII using HPLC. Lineweaver-Burk plots of the

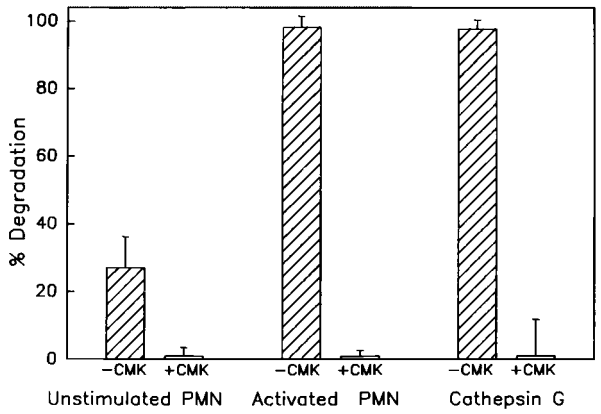

FIGURE 1. ANGI-converting activity of membrane-bound cathepsin G. Unstimulated PMN and activated PMN were fixed, then cells (10^7/assay) or soluble cathepsin G (50 nM) were incubated with 100 nM human ANGI in the presence and the absence of the specific cathepsin G inhibitor CMK (600 μM). Disappearance of ANGI was quantified in triplicate by RIA. Note that free cathepsin G and activated PMN nearly completely degraded the ANGI, and that this activity was completely inhibited by the cathepsin G inhibitor. In contrast, unstimulated PMN had much more modest activity. Data are mean values; error bars represent SDs.

reciprocals of the reaction velocity and substrate concentrations were constructed, and the Michaelis Menten constant (K_m) and maximum reaction velocity (V_{max}) were calculated.

Inhibition of free and cell surface-bound cathepsin G

Catalytically equivalent amounts (40 nM) of membrane-bound cathepsin G on fixed activated PMN or of free cathepsin G were preincubated with various inhibitors for 20 min at 37°C, following which ANGI (300 μM) was added for an additional 45 min. Residual enzyme activity was inhibited by the addition of PMSF (500 μM), and cell-free supernatant fluids were assayed in triplicate for ANGII by HPLC.

To determine the IC_{50} for membrane-bound vs free cathepsin G for α_1-antichymotrypsin and SLPI, catalytically equivalent amounts of membrane-bound and free cathepsin G were incubated for 2 h at 37°C with and without varying concentrations of inhibitor. To study the effectiveness of up to a 10^5-fold molar excess of α_1-antichymotrypsin and up to a 10^3-fold molar excess of SLPI over membrane-bound cathepsin G, we studied 1 and 10 nM enzyme, respectively. ANGI (300 μM) was added, and the samples were incubated for up to 150 min. PMSF (1 mM) was added, and cell-free supernatant fluids were assayed in triplicate for ANGII by HPLC. The IC_{50} values were determined by linear regression analysis.

The capacities of free and cell surface-bound cathepsin G to generate ANGII in the presence of plasma proteinase inhibitors were compared. Human ANGI (300 μM) was added to normal human plasma along with pepstatin A (50 μM), captopril (1 mM), and 8-hydroxyquinoline (0.66 μg/ml) to inhibit endogenous renin, angiotensin-converting enzymes, and angiotensinases, respectively. Fixed PMN (10^7/ml), free cathepsin G (40 nM), or HBSS (as a control) were added to the plasma (300 μl) and incubated for 60 min at 37°C. After addition of PMSF (500 μM), cell-free supernatant fluids were harvested, then ANGII was extracted essentially as described previously (34). Briefly, the samples were applied to Supelco solid phase phenyl-silica extraction tubes (Bellefonte, PA) and washed with 1 ml of distilled H_2O followed by 1 ml of methanol. The samples were collected and dried overnight in a Speed-Vac, then resuspended in 100 μl of solvent and assayed for ANGII by HPLC.

Results

To investigate the ANGII-generating activity of membrane-bound enzymes on human PMN, it was necessary to study cells that had been fixed to prevent leakage of intracellular serine proteinases (26). Hereafter, PMN and PBMC refer to cells that have been fixed, as described in *Materials and Methods*.

Membrane-bound cathepsin G on activated PMN converts ANGI to ANGII

To test the possibility that cell surface-bound cathepsin G is catalytically active against ANGI, unstimulated PMN and activated

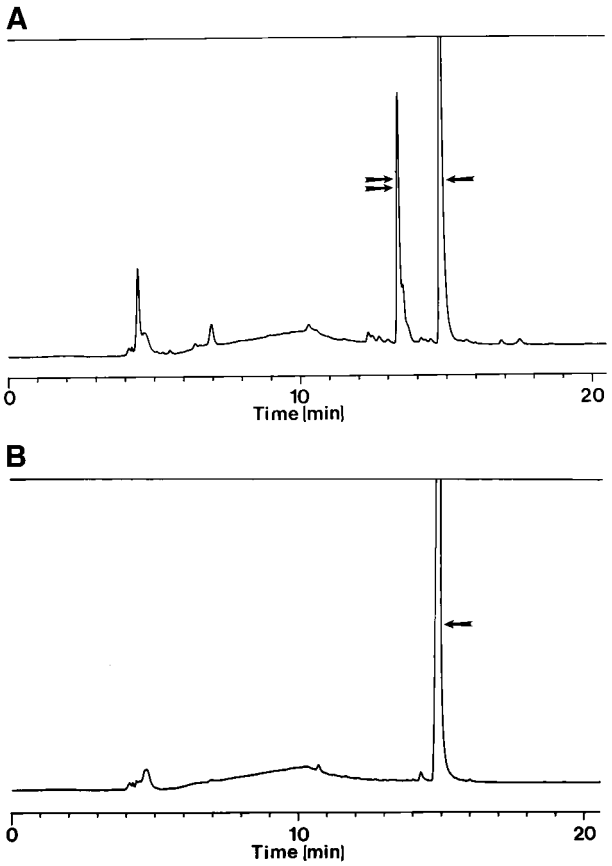

FIGURE 2. Activated PMN convert ANGI to ANGII. *A*, Fixed activated PMN (10^7) were incubated with 300 μM ANGI for 45 min at 37°C. *B*, As a control, ANGI was incubated in the absence of cells. Cell-free supernatant fluids were subjected to reverse phase HPLC as described in *Materials and Methods*. Note that ANGII (double arrow) is the major degradation product when activated cells are incubated with ANGI (single arrow).

PMN as well as free cathepsin G were incubated for 2 h at 37°C with purified human ANGI. Free cathepsin G and activated PMN completely degraded ANGI, whereas unstimulated PMN were much less active and only partially degraded ANGI (Fig. 1).

To confirm that activated PMN convert ANGI to ANGII, the reaction products were applied to reverse phase HPLC. When activated PMN were incubated with purified ANGI, ANGII was clearly detected in cell-free supernatant fluids (Fig. 2*A*), but no ANGII was found in the absence of cells (Fig. 2*B*). The ANGII-generating activity expressed by activated PMN was clearly cell associated, since cell-free supernatant fluids from activated cells analyzed by HPLC produced no detectable ANGII-generating activity when tested against ANGI (not shown).

To test the possibility that ANGII-generating activity expressed by activated PMN is mediated by membrane-bound cathepsin G, we assessed the effect of CG/CMK, a specific synthetic inhibitor of cathepsin G. The ANGI-degrading activity that was expressed by free cathepsin G and PMN was completely inhibited by CG/CMK (Fig. 1). The ANGII-generating activity expressed by activated PMN could not be attributed to the release of intracellular cathepsin G, since no cathepsin G activity was detected in cell-free supernatant fluids using SAAF-AFC, a fluorogenic substrate that is specific for cathepsin G (not shown).

To assess the ability of membrane-bound cathepsin G on activated PMN to convert angiotensinogen to ANGII, human angiotensinogen (100 μM) was incubated for 30 min at 37°C with and

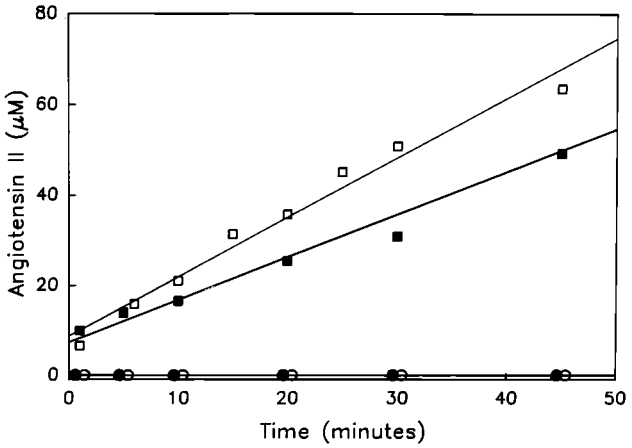

FIGURE 3. Human leukocyte elastase and proteinase 3 do not contribute to the ANGII-generating activity of activated PMN. Catalytically equivalent amounts (20 nM) of purified human leukocyte elastase (open circles), purified proteinase 3 (closed circles), purified cathepsin G (closed squares), or membrane-bound cathepsin G on fixed activated PMN (open squares) were incubated at 37°C with 300 μM ANGI for varying times as described in *Materials and Methods*. Cell-free supernatant fluids were assayed in triplicate for ANGII by HPLC. Note the progressive accumulation of ANGII in reactions containing purified cathepsin G and activated PMN. In marked contrast, no ANGII was generated by human leukocyte elastase or proteinase 3. Data are mean values; error bars (which are smaller than the symbols) represent SDs.

without activated cells (10^7/ml) or soluble cathepsin G (40 nM), then ANGII was quantified in cell-free supernatant fluids by HPLC. Free cathepsin G and activated cells generated similar (but small) quantities of ANGII (700 and 800 nM, respectively). No ANGII was detected in the absence of cells or soluble cathepsin G. Together, these data indicate that cathepsin G expressed on the cell surface of activated PMN is active against both angiotensinogen and ANGI, and that ANGII is a major reaction product.

PMN surface-bound human leukocyte elastase, proteinase 3, and EC 3.4.15.1 do not contribute to the ANGII-generating activity that is expressed by activated PMN

Activated PMN have been shown to express cell surface-bound human leukocyte elastase and proteinase 3 (26, 35, 36). To provide assurance that the ANGII-generating activity that is expressed by activated PMN is due to membrane-bound cathepsin G and not due to membrane-bound elastase or proteinase 3, catalytically equivalent amounts of these serine proteinases or activated PMN were incubated with ANGI for up to 45 min, then ANGII generation was quantified by HPLC. ANGII progressively accumulated only in the presence of cathepsin G or activated PMN (Fig. 3).

To exclude the possibility that activation of PMN induces cell surface expression of dipeptidyl carboxypeptidase angiotensin-converting enzyme (EC 3.4.15.1), unstimulated and PAF-primed and FMLP-stimulated PMN were assayed for this activity, and none was detected (not shown).

To provide further assurance that PMN ANGII-generating activity is mediated by membrane-bound cathepsin G, peripheral blood PBMC, which express no cell surface cathepsin G, leukocyte elastase, or EC 3.4.15.1 under basal conditions (37), were studied. PBMC were fixed, then incubated with and without exogenous cathepsin G or human leukocyte elastase, which bind readily to the surface of fixed cells (26). The cells were washed to remove unbound enzyme, then fixed again. PBMC that were incubated with human leukocyte elastase and cathepsin G expressed 300.8 ± 8.0 and 358.2 ± 5.4 (±SD) ng of enzyme/10^6 cells, respectively, when tested against synthetic fluorogenic substrates that are specific for these serine proteinases. When the cells were incubated for 20 min with ANGI (300 μM), PBMC that were incubated without exogenous enzyme and cells that were incubated with human leukocyte elastase generated no ANGII that was detectable by HPLC. In contrast, cells that had been incubated with cathepsin G generated 17.1 ± 1.2 μM ANGII/10^6 cells. Together, these data confirm that membrane-bound cathepsin G mediates the ANGII-generating activity that is expressed by activated PMN.

Table I. *Quantification of catalytically active membrane-bound cathepsin G expressed by activated PMN*

Donor[a]	Cathepsin G Activity	
	SAAF-AFC[b]	HPLC[c]
1	154.0 ± 6.9[d]	145.5 ± 4.9
2	186.2 ± 7.0	196.1 ± 0.8
3	320.2 ± 4.2	285.3 ± 3.6
4	210.7 ± 6.7	229.0 ± 1.7
Mean (SD)	217.8 ± 72.1	214.0 ± 58.7

[a] PMN from four donors were primed for 1 min at 37°C with PAF (10^{-9} M), then stimulated for 30 min with FMLP (10^{-8} M). Cells were fixed with 3% paraformaldehyde and 0.5% glutaraldehyde.
[b] Cathepsin G activity expressed by fixed activated cells was assayed in triplicate using SAAF-AFC, as described in *Materials and Methods.*
[c] Cathepsin G activity expressed by fixed activated cells was assayed in triplicate by HPLC using ANGI as the substrate, as described in *Materials and Methods.*
[d] Data are ng of cathepsin G per 10^6 cells (mean ± SD).

Quantification of catalytic activity of cathepsin G bound to activated PMN

To quantify the amount of catalytically active cathepsin G that is expressed on the surface of activated PMN, activated PMN from four donors or varying amounts of purified cathepsin G were assayed against ANGI and SAAF-AFC, a fluorogenic substrate that is specific for cathepsin G. Activated PMN had similar activities against both substrates (Table I). The cells expressed a mean of 214.0 ± 58.7 ng of cathepsin G activity against ANGI/10^6 cells, which is equivalent to 8.56 ± 2.3 × 10^{-18} mol (or 5.16 ± 1.39 × 10^6 molecules) of active cathepsin G/cell.

Kinetics of ANGII generation by free vs membrane-bound cathepsin G

To determine saturation kinetic constants, catalytically equivalent amounts (20 nM) of free and membrane-bound CG were incubated with 50 to 800 μM ANGI in HBSS for 15 min at 37°C, then cell-free supernatant fluids were assayed for ANGII by HPLC. Lineweaver-Burk plots demonstrated saturation kinetics (Fig. 4). The K_m values for free and membrane-bound cathepsin G were 4.6 × 10^{-4} and 5.9 × 10^{-4} M, respectively, and the V_{max} values were 58.0 and 28.1 nmol of ANGII generated/h, respectively. The k_{cat}, calculated as V_{max} per picomole of cathepsin G, was 4.0/s for free cathepsin G and 2.0/s for membrane-bound enzyme. Due to the low level catalytic activity of both free and membrane-bound cathepsin G against angiotensinogen, it was not possible to determine saturation kinetics for this substrate.

ANGII-generating activity expressed by cell surface-bound cathepsin G is resistant to inhibition by naturally occurring proteinase inhibitors

Free and membrane-bound cathepsin G on PMN were incubated with ANGI with and without various proteinase inhibitors of varying size, then ANGII generation was quantified in the cell-free supernatant fluids by HPLC. The cathepsin G inhibitors were fully

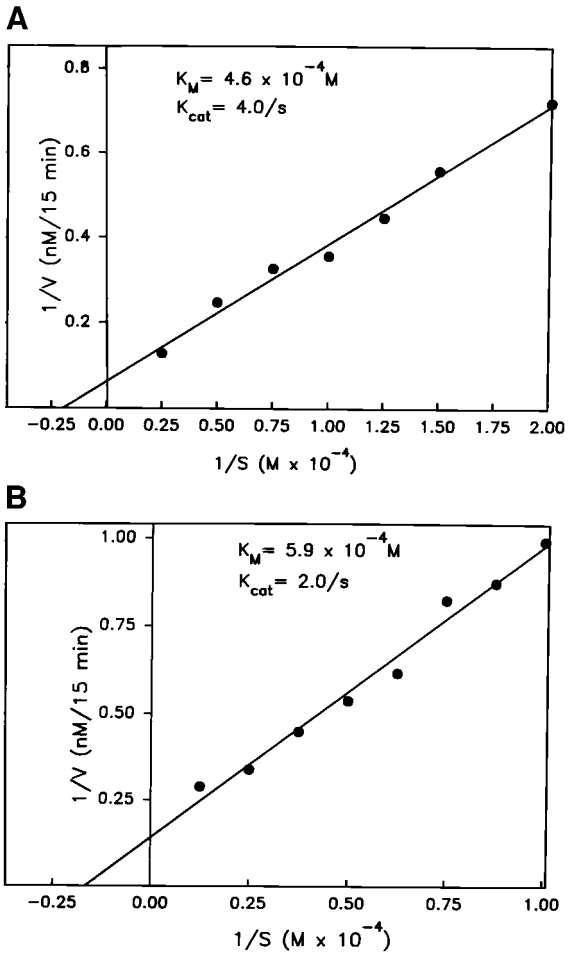

FIGURE 4. Lineweaver-Burk analysis of ANGII generation by soluble and membrane-bound cathepsin G. Catalytically equivalent amounts (20 nM) of free (*A*) and membrane-bound (*B*) cathepsin G were incubated for 15 min at 37°C with 50 to 800 μM ANGI, then cell-free supernatant fluids were analyzed in triplicate by HPLC. Note that the K_m for free cathepsin G was similar to that for membrane-bound enzyme.

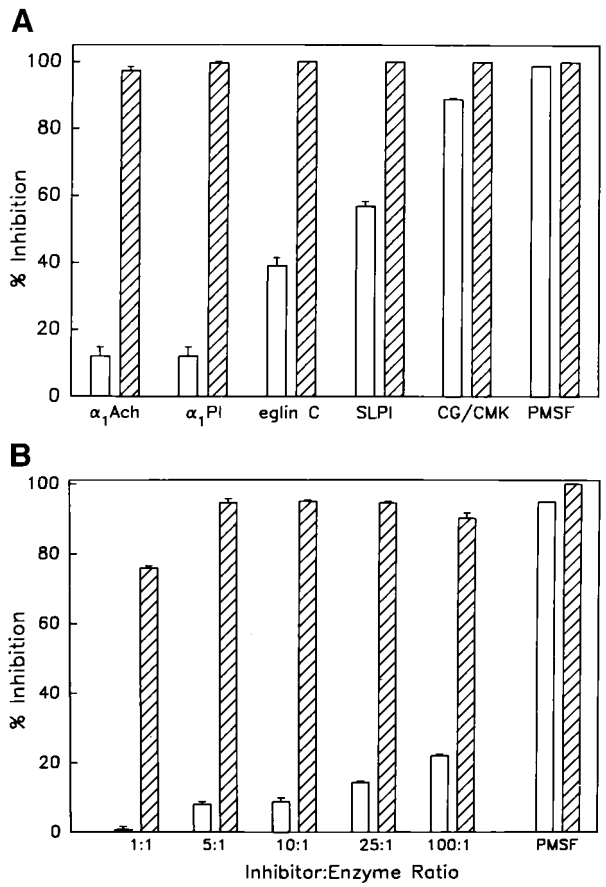

FIGURE 5. Effect of proteinase inhibitors on soluble vs membrane-bound cathepsin G. Cross-hatched bars show free cathepsin G; open bars show membrane-bound cathepsin G. *A*, Catalytically equivalent amounts (40 nM) of free and membrane-bound cathepsin G on fixed activated PMN were incubated for 20 min at 37°C with and without 1) α_1-antichymotrypsin (α_1Ach; 1 μM), 2) α_1-proteinase inhibitor (α_1PI; 1 μM), 3) eglin C (1 μM), 4) SLPI (1 μM), 5) CG/CMK (1 mM), and 6) PMSF (1 mM). The samples were then incubated with 300 μM ANGI, then cell-free supernatant fluids were assayed in triplicate for ANGII. Note that all the inhibitors were effective against free cathepsin G. PMSF and CG/CMK were also effective against membrane-bound enzyme. In marked contrast, α_1-antichymotrypsin and α_1-proteinase inhibitor produced minimal inhibition of membrane-bound cathepsin G. Eglin C and SLPI had intermediate effectiveness against membrane-bound cathepsin G. *B*, Catalytically equivalent amounts (40 nM) of free and membrane-bound cathepsin G were incubated for 20 min at 37°C with and without varying amounts of α_1-antichymotrypsin (α_1Ach), ranging from equimolar to a 100-fold excess, or PMSF (1 mM). The samples were incubated with ANGI (300 μM), then cell-free supernatant fluids were assayed in triplicate by HPLC. Note that α_1-antichymotrypsin was only partially effective against membrane-bound cathepsin G even when present in substantial molar excess. Data are mean values; error bars represent SDs.

effective, at the concentrations tested, as inhibitors of free cathepsin G (Fig. 5*A*). In contrast, an inhibitor of human leukocyte elastase (HLE/CMK) and captopril, a potent inhibitor of dipeptidyl carboxypeptidase, both had minimal effects on free cathepsin G (3.8 ± 0.4 and 8.8 ± 0.2% (±SD) inhibition, respectively).

α_1-Antichymotrypsin and α_1-proteinase inhibitor, which are high affinity, naturally occurring inhibitors of free cathepsin G, were strikingly ineffective (12 and 12% inhibition, respectively) against PMN membrane-bound cathepsin G activity (Fig. 5*A*). Moreover, α_1-antichymotrypsin, the cognate inhibitor of cathepsin G, was ineffective against membrane-bound cathepsin G even when added at a 100-fold molar excess (Fig. 5*B*). In marked contrast, the low m.w. proteinase inhibitors PMSF and CG/CMK almost completely inhibited membrane-bound cathepsin G (Fig. 5*A*). SLPI and eglin C, which are of intermediate m.w., were only moderately effective (57 and 39% inhibition, respectively). HLE/CMK and captopril had minimal effect on membrane-bound cathepsin G (5.0 ± 0.1 and 2.7 ± 1.4% inhibition, respectively).

To confirm that activated PMN can convert ANGI in inhibitor-containing biologic fluids, activated PMN or free cathepsin G were incubated with undiluted human plasma that contained added human ANGI. Activated PMN converted ANGI to ANGII in the presence of proteinase inhibitors contained within plasma, but there was no detectable ANGII generation by free cathepsin G (Fig. 6).

Exposure of PMN to fixatives could potentially reduce the effectiveness of high m.w. proteinase inhibitors against membrane-bound cathepsin G. Thus, we tested the efficacies of various inhibitors against exogenously added cell surface cathepsin G that had not been exposed to fixatives. α_1-Antichymotrypsin, eglin C, and PMSF had similar effectiveness against unfixed, exogenously added, membrane-bound cathepsin G (16.8 ± 0.1, 41.6 ± 0.3, and 97.4 ± 0.2%, respectively) compared with membrane-bound enzyme on fixed PMN (Fig. 5*A*). These data confirm that, as for membrane-bound human leukocyte elastase (26), exposure of membrane-bound cathepsin G to fixatives does not adversely affect its interaction with proteinase inhibitors.

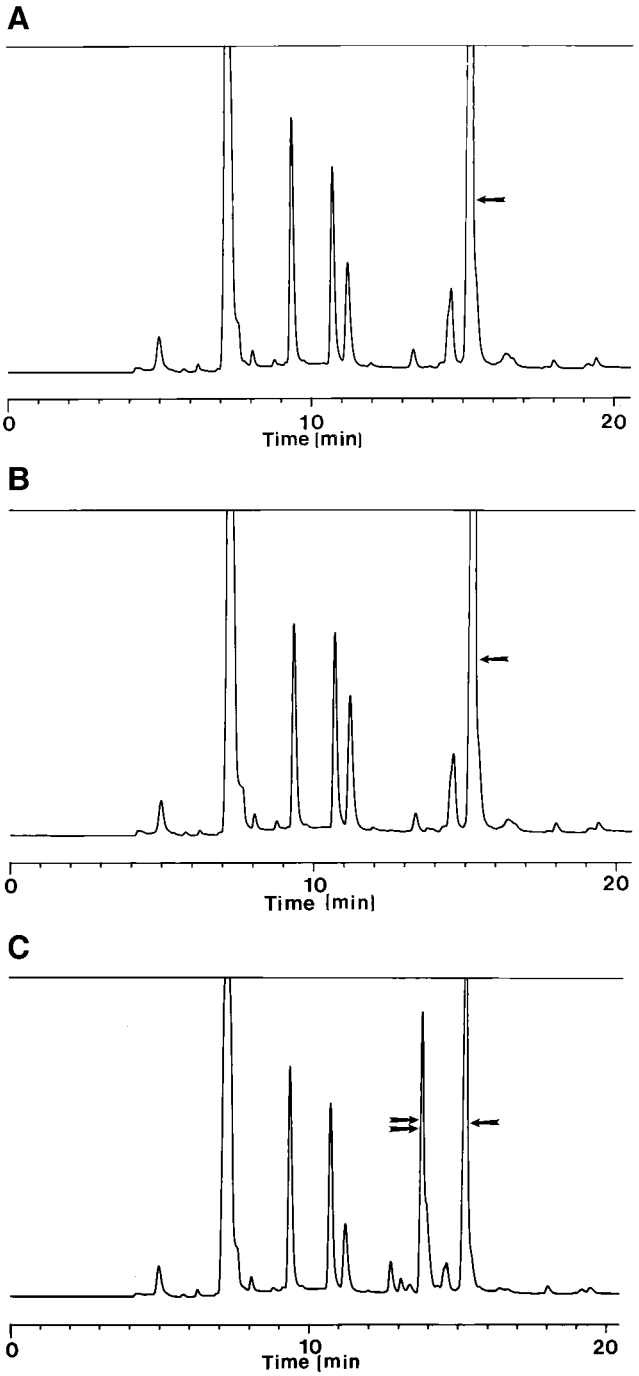

FIGURE 6. Effects of plasma proteinase inhibitors on ANGII generation by membrane-bound cathepsin G. Human plasma was spiked with 300 μM human ANGI (single arrow) and with pepstatin A, captopril, and 8-hydroxyquinoline to inhibit endogenous renin, angiotensin-converting enzymes, and angiotensinases. Fixed activated PMN (10^7/ml) or free cathepsin G (40 nM) were incubated with the plasma for 60 min at 37°C. PMSF was added to terminate the reaction, then cell-free supernatant fluids were analyzed by HPLC. Note that ANGII was not detected in plasma that was incubated in the absence of cells or free cathepsin G (*A*) or in the presence of free cathepsin G (*B*). In marked contrast, PMN generated ANGII (double arrow) in the presence of plasma proteinase inhibitors (*C*).

Inhibitor kinetics

The ability of α_1-antichymotrypsin and SLPI to inhibit free vs membrane-bound cathepsin G as a function of time revealed complete inhibition of free cathepsin G by equimolar amounts of α_1-antichymotrypsin or SLPI within 20 min. In marked contrast, incubation of membrane-bound cathepsin G with equimolar amounts of α_1-antichymotrypsin or SLPI for 8 h resulted in only 2.7 $\pm$ 0.4 and 9.9 $\pm$ 11.8% inhibition of membrane-bound cathepsin G activity, respectively. Due to the lack of complete inhibition of membrane-bound cathepsin G by α_1-antichymotrypsin and SLPI, it was not possible to determine second order association rate constants for this form of the enzyme and these inhibitors.

Since it was not possible to determine association rate constants for membrane-bound cathepsin G and biologically relevant proteinase inhibitors, we measured inhibition as a function of inhibitor concentration for α_1-antichymotrypsin and SLPI. The IC_{50} value for SLPI when tested against 10 nM free cathepsin G was 15.2 nM, whereas the IC_{50} for the same inhibitor against 10 nM membrane-bound enzyme (2.24 μM) was nearly 150-fold greater (Fig. 7*A*). An even more remarkable difference (>1580-fold) in the IC_{50} value was found for α_1-antichymotrypsin (Fig. 7*B*) when tested against 1 nM free or membrane-bound cathepsin G (1.38 nM and 2.18 μM, respectively). It is especially noteworthy that it was not possible to achieve complete inhibition of membrane-bound cathepsin G activity even when α_1-antichymotrypsin was present at a 10^5-fold molar excess over enzyme. These data confirm that membrane-bound cathepsin G is substantially resistant to inhibition by naturally occurring inhibitors and that there is a striking inverse relationship between inhibitor size and its effectiveness against membrane-bound cathepsin G activity.

Discussion

Herein, we report that cathepsin G that is expressed on the surface of activated PMN has potent ANGII-generating activity. Membrane-bound cathepsin G has very high affinity ($K_m = \sim 10^{-4}$ M) for ANGI, but this activity is remarkably resistant to inhibition by plasma proteinase inhibitors. Together, these data indicate that ANGII-generating activity that is expressed at the cell surface of activated PMN may play important local vasoregulatory roles at sites of inflammation.

Membrane-bound cathepsin G has potent ANGII-generating activity

HPLC was used to quantify ANGII generation by free cathepsin G and by activated PMN. Other laboratories have previously validated this technique for quantifying cathepsin G-mediated generation of ANGII from ANGI (21, 22). Our data indicate that activated PMN convert ANGI to ANGII in a time-dependent manner, and that (like soluble cathepsin G (20–22)), activated PMN also convert angiotensinogen directly to ANGII, albeit at a much slower rate.

Several lines of evidence confirmed that the ANGII-generating activity that is expressed by activated PMN is mediated by membrane-bound cathepsin G. First, PMN-associated ANGII-generating activity was completely inhibited by a specific inhibitor of cathepsin G (CG/CMK), whereas a control inhibitor (HLE-CMK) was completely ineffective. Second, PMN do not express other enzymes that have ANGII-generating activities such as chymase (38, 39) or EC 3.4.15.1, and ANGII generation by PMN is not inhibited by captopril, an effective inhibitor of the latter enzyme. Third, ANGII-generating activity can readily be conferred upon PBMC (which have no endogenous basal activity) (37) by the binding of exogenous cathepsin G, but not human leukocyte elastase, to these cells. Fourth, other serine proteinases that are expressed on the cell surface of activated PMN (human leukocyte elastase and proteinase 3) do not have the capacity to convert ANGI to ANGII. Finally, activated PMN from four different donors express almost identical catalytic activity when tested against

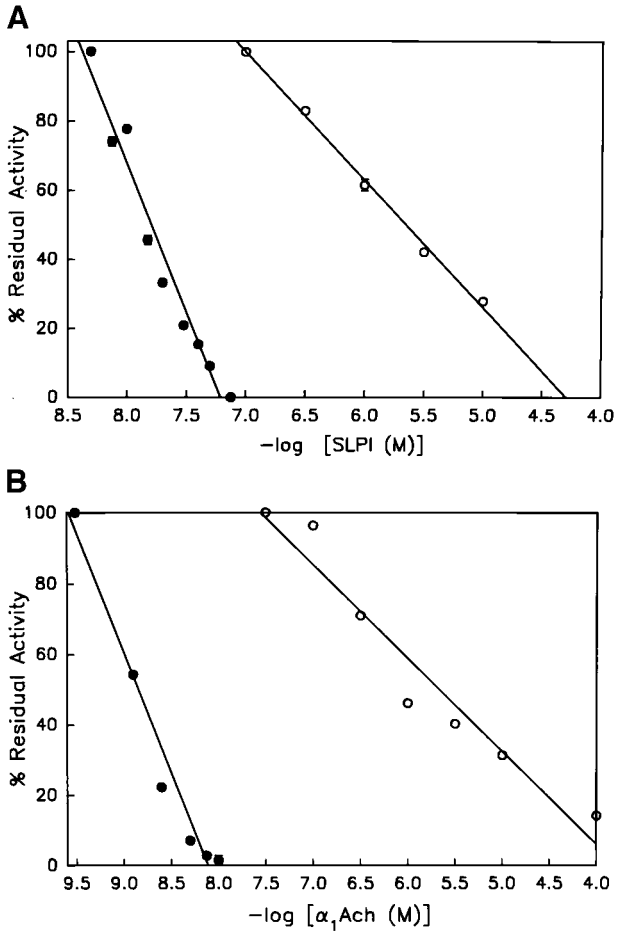

FIGURE 7. Concentration dependence of inhibition of membrane-bound cathepsin G by SLPI and α_1-antichymotrypsin. *A,* Catalytically equivalent amounts (10 nM) of free (solid circles) and membrane-bound cathepsin G on activated PMN (open circles) were preincubated for 2 h at 37°C with varying concentrations of SLPI, then incubated for 60 min with ANGI (300 μM). *B,* Catalytically equivalent amounts (1 nM) of free (solid circles) and membrane-bound cathepsin G on activated PMN (open circles) were preincubated for 150 min at 37°C with varying concentrations of α_1-antichymotrypsin. The samples were then incubated with ANGI (300 μM) for 150 min. ANGII was quantified in triplicate in cell-free supernatant fluids by HPLC, and IC_{50} values were determined by linear regression analysis. Note that the IC_{50} values for membrane-bound cathepsin G are substantially greater than those for free cathepsin G.

ANGI and SAAF-AFC, a synthetic fluorogenic substrate that is specific for cathepsin G.

When ANGI was used as the substrate, the K_m and k_{cat} values for membrane-bound cathepsin G were similar to those for free enzyme and are the highest known for this enzyme (22, 40). The latter observation suggests that local generation of ANGII from ANGI at the surface of PMN is a biologically important function of membrane-bound cathepsin G on PMN in vivo.

Membrane-bound cathepsin G is resistant to inhibition by proteinase inhibitors

When inhibitors of cathepsin G with a range of molecular sizes were tested, all the inhibitors were effective against free cathepsin G; however, there was a striking indirect relationship between the size of the inhibitor and its effectiveness against membrane-bound cathepsin G. Of particular biologic interest was the observation that high m.w. naturally occurring inhibitors such as α_1-antichymotrypsin and α_1-proteinase inhibitor were almost completely ineffective against membrane-bound cathepsin G. When concentration dependence of inhibition was assessed for an intermediate-sized inhibitor (SLPI) and α_1-antichymotrypsin, the IC_{50} values were ~150- and ~1600-fold higher, respectively, for membrane-bound cathepsin G compared with those for free enzyme. It is noteworthy that incomplete inhibition of membrane-bound cathepsin G was observed when α_1-antichymotrypsin was present at 10^5-fold molar excess over enzyme, and that activated PMN generated ANGII even in undiluted human plasma. In marked contrast, free cathepsin G was completely inhibited by plasma proteinase inhibitors. Together, these data indicate that membrane-bound cathepsin G expressed by activated PMN at sites of inflammation in vivo is likely to be persistently active despite the presence of high affinity inhibitors of free cathepsin G.

Because of the strong inverse relationship between the m.w. of the inhibitor and its capacity to inhibit membrane-bound cathepsin G, it is likely that steric hindrance is the major mechanism by which membrane-bound cathepsin G evades inhibition. This hypothesis is supported by the observation that membrane-bound cathepsin G was substantially less catalytically active against angiotensinogen (50 kDa) compared with ANGI (1.2 kDa).

We did not directly test the effectiveness of α_2m against membrane-bound and purified cathepsin G because the substrate of interest (ANGI) is small enough to gain access to enzymes entrapped within α_2m (41). However, the very large size of α_2m and its mechanism of action argue that this inhibitor would be an ineffective inhibitor of membrane-bound cathepsin G.

The mechanism by which cathepsin G binds to the PMN cells surface was not investigated herein. However, preliminary Scatchard analysis using radiolabeled cathepsin G indicate that PMN express high volume, low affinity binding sites for cathepsin G (C. A. Owen and E. J. Campbell, manuscript in preparation). Moreover, we have shown previously that cathepsin G can be eluted from the PMN plasma membrane by exposure to solutions with high ionic strength (25). These observations together with the fact that cathepsin G is a highly cationic molecule suggest that cathepsin G binds to negatively charged plasma membrane constituents. This possibility is the focus of ongoing studies in our laboratory.

Potential biologic importance of ANGII generation by membrane-bound cathepsin G

There has been a great deal of speculation about the biologic roles of cathepsin G, since minimal quantities are freely released from PMN in response to biologically relevant stimuli (24, 25), and the extracellular space is replete with high affinity inhibitors of this enzyme (41). We have shown that membrane-bound cathepsin G on PMN is readily accessible to (and has high affinity for) ANGI, yet is resistant to inhibition by plasma proteinase inhibitors. These results indicate that one potential role for cathepsin G is to locally generate ANGII at or near the cell surface of PMN during the inflammatory response.

The specificity constant (k_{cat}/K_m) for membrane-bound cathepsin G for ANGI is the highest known for the enzyme (calculated as $6.8 \times 10^3\ M^{-1}\ s^{-1}$); however, it is significantly lower than that for dipeptidyl carboxypeptidase and ANGI ($1.9 \times 10^5\ M^{-1}\ s^{-1}$) (42, 43) and even lower than that for human heart chymase and ANGI ($2.7 \times 10^6\ M^{-1}\ s^{-1}$) (14, 15). Thus, membrane-bound cathepsin G may not contribute significantly to ANGII generation in plasma or heart. This concept is supported by the observations that 1) ANGII formation in normal serum can be completely inhibited by captopril (44); and 2) the majority of ANGII-forming activity in

the human left ventricle is not inhibited by aprotinin, which inhibits cathepsin G but not chymase (44). However, in contrast to the dipeptidyl carboxypeptidase and cardiac chymase systems, membrane-bound cathepsin G on activated PMN is an ANGII-generating system that is both highly mobile and readily inducible by proinflammatory mediators. PMN-mediated ANGII generation can thus be targeted to and markedly up-regulated at inflammatory foci. Membrane-bound cathepsin G on activated PMN sequestered at sites of inflammation may be an important pathway for the local production of ANGII in diseased tissue, where it may play important roles in the enhancement of vascular permeability and cellular infiltration.

Conclusions

We have demonstrated that membrane-bound cathepsin G on activated PMN is a novel pathway for generating ANGII at the cell surface of PMN at sites of inflammation. Membrane-bound cathepsin G has high affinity for ANGI, and this form of the enzyme is remarkably resistant to inhibition by plasma proteinase inhibitors. Together, these data suggest that membrane-bound cathepsin G is the bioactive form of the enzyme in vivo, and that local generation of ANGII at the cell surface of PMN during the inflammatory response is an important function of membrane-bound cathepsin G. Based on the known biologic activities of ANGII, activated PMN in the vascular space are likely to play important roles in local vasoregulation and vascular permeability and in modulating immune and inflammatory reactions at sites of infection and inflammation in vivo.

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