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ESSENTIAL ROLE FOR CATHEPSIN D IN BLEOMYCIN-INDUCED APOPTOSIS OF ALVEOLAR EPITHELIAL CELLS

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Running Head: Cathepsin D in Alveolar Epithelial Apoptosis

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ABSTRACT

Earlier studies from this laboratory showed that bleomycin-induced apoptosis of type II alveolar epithelial cells (AECs) requires the autocrine synthesis and proteolytic processing of angiotensinogen into angiotensin II (ANGII), and that inhibitors of ANG converting enzyme (ACEis) block bleomycin-induced apoptosis (Li, X, H Zhang, V Soledad-Conrad, J Zhuang and BD Uhal. Bleomycin-induced apoptosis of alveolar epithelial cells requires angiotensin synthesis *de novo*. Am. J. Physiol. 284(3):L501-L507, 2003.). Given the documented role of cathepsin D (CatD) in apoptosis of other cell types, we hypothesized that CatD might be the AEC enzyme responsible for the conversion of angiotensinogen into ANGI, the substrate for ACE. Primary cultures of rat type II AECs challenged with bleomycin *in vitro* showed upregulation and secretion of CatD enzymatic activity and immunoreactive protein, but no increases in CatD mRNA. The aspartyl protease inhibitor pepstatin A, which completely blocked CatD enzymatic activity, inhibited bleomycin-induced nuclear fragmentation by 76% ($p<0.01$) and reduced bleomycin-induced caspase 3 activation by 47% ($p<0.05$). Antisense oligonucleotides against CatD mRNA reduced CatD immunoreactive protein and inhibited bleomycin-induced nuclear fragmentation by 48% ($p<0.01$). A purified fragment of angiotensinogen (F1-14) containing the CatD and ACE cleavage sites, when applied to unchallenged AEC *in vitro*, yielded mature ANGII peptide and induced apoptosis. The apoptosis induced by F1-14 was inhibited 96% by pepstatin A and 77% by neutralizing antibodies specific for CatD (both $p<0.001$). These data indicate a critical role for CatD in bleomycin-induced apoptosis of cultured AEC, and suggest that the role(s) of CatD in AEC apoptosis include the conversion of newly synthesized angiotensinogen to ANGII.

Key Words:

aspartyl protease lung injury programmed cell death

lung fibrosis type II pneumocyte

INTRODUCTION

Angiotensin II (ANGII) is a potent inducer of apoptosis in alveolar epithelial cells (AEC) and is synthesized and released from AEC undergoing apoptosis in response to other stimuli (3). Work from this laboratory has shown that ANGII is secreted by AEC challenged in vitro with Fas ligand (15) or TNF-alpha (14), and demonstrated that the production of ANGII is required for the signaling of apoptosis by these inducers. More recent investigations showed that AEC apoptosis in response to the fibrogenic agent bleomycin requires the autocrine synthesis of angiotensin II (ANGII) and the subsequent binding of ANGII to receptor subtype AT1 (8). Apoptosis of AEC in response to the fibrogenic antiarrhythmic agent amiodarone also is blocked by ANG receptor AT1-selective antagonists (3,13). Together, these findings have lead to the theory that autocrine production of ANGII by AEC *de novo*, i.e., from the precursor angiotensingen, is a common event required for AEC apoptosis regardless of the initiating stimulus (11).

Primary cultures of rat AEC were shown to constitutively express low but functional levels of angiotensin converting enzyme (ACE) mRNA (16), and to respond to ACE inhibitors such as captopril or lisinopril (12,14). However, the identity of the enzyme(s) in AEC which act upstream of the ACE reaction, i.e., performing the conversion of angiotensinogen to ANGI (the substrate for ACE), remains unknown. In the serum, the conversion of liver-derived circulating angiotensinogen into ANGI is accomplished by the kidney-derived enzyme renin; this system is now viewed as the classical “endocrine” renin-angiotensin system (2). In contrast, tissue-specific “local” angiotensin systems exist in many tissues as either “extrinsic” systems (i.e., dependent on one or more components of the endocrine system) or as “intrinsic” ANG systems in which all the enzymes and substrates required for the production of ANGII are synthesized locally. In local

intrinsic systems outside the lung, the primary aspartyl protease that converts newly synthesized angiotensinogen to ANGI is cathepsin D (CatD, 17), a ubiquitous lysosomal aspartyl protease expressed by virtually all cells (5). The identity of this aspartyl protease in the pulmonary local ANG system is the subject of this study.

A critical role for CatD in the execution of apoptosis has been shown previously in a variety of cell types including kidney cell lines (4), PC12 cells and dorsal root ganglion-derived neurons (5) and in ML1 leukemia or U1752 lung cancer cells exposed to etoposide or adriamycin (18). In those studies, apoptosis in response chemical stimuli or trophic withdrawal could be prevented by the aspartyl protease inhibitor pepstatin A or by antisense oligonucleotides against CatD mRNA. One investigation of CatD-dependent apoptosis in neuronal cells led to the hypothesis that activation of CatD by apoptosis inducers leads to the generation of an unidentified “bioactive molecule” that is required for the signaling of apoptosis, but is either degraded or expressed at low levels in viable cells under basal unstimulated conditions (5).

In the light of previous demonstrations that AEC apoptosis requires the autocrine synthesis of ANGII and the documented ability of CatD to convert angiotensinogen to ANGI, we hypothesized that CatD might be required for AEC apoptosis. We also theorized that the primary function of CatD in AEC apoptosis is the conversion of angiotensinogen to ANGI. We report here that AEC apoptosis in response to bleomycin is inhibited by CatD knockdown as a result of its blockage of ANGII synthesis.

MATERIALS AND METHODS

Reagents and materials: The ANG receptor AT1-selective antagonist L158809 was obtained from Merck and Co., West Point, PA. The CatD fluorescent substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 (19) was obtained from Peptides International, Louisville, KY. Bleomycin (BLEO), anti-angiotensin antibodies, ATA, captopril and saralasin were obtained from Sigma Chemical Co., Saint Louis , MO. A kit for ELISA quantitation of angiotensin II was obtained form Peninsula Laboratories (San Carlos, CA). All other materials were of reagent grade and were obtained from Sigma Chemical Co., Saint Louis, MO.

Cell culture: Primary alveolar epithelial cells isolated from adult male Wistar rats as described earlier (15). The primary cells were studied at day two of culture, a time at which they are type II cell-like by accepted morphologic and biochemical criteria (9). Primary cell preparations were of better than 90% purity assessed by acridine orange staining as described previously (14,15). All cells were grown in 24- or 6-well chambers and were analyzed at subconfluent densities of 80-90%. All subsequent incubations with BLEO and/or other test agents were performed in serum-free medium. The cells were exposed to caspase inhibitors or antagonists of the angiotensin system 30 minutes before exposure to BLEO for 1-20 hours as indicated.

Quantitation of nuclear fragmentation and caspase 3 activity: Detection of apoptotic cells with propidium iodide (PI) was conducted as described earlier (14,15) following digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5ug/ml PI. In these assays, detached cells were retained by centrifugation of the 24-well culture vessels during fixation with 70% ethanol. Cells with discrete nuclear fragments containing condensed chromatin were scored

as apoptotic. As in earlier publications, the induction of apoptosis was verified by in situ end labeling (ISEL) of fragmented DNA (8). Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group.

The enzymatic activity of Caspase 3 was measured in adherent cells incubated for 20 hours with the membrane permeable substrate Ac-DEVD-AMC (Upstate Biotech, Saranac Lake, NY) at 50uM final concentration. Quantitation of the fluorescent product was achieved with a Biotek FL600 fluorescence plate reader. Fluorescence values were normalized to cell number determined on the same culture well after cell fixing and staining of DNA with PI (15).

Assay of CatD activity: The enzymatic activity of CatD was determined with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 as described by its inventors (19). Briefly, aliquots of AEC lysates or concentrated cell culture media were incubated in opaque 96-well culture plates (suitable for top reading in a fluorescence plate reader) in 1.0M sodium acetate buffer, pH 4.0 containing 50uM fluorogenic substrate. The total volume of reaction buffer, including sample, was 100ul. In the case of cell lysates, equal amounts of lysate protein were assayed per culture vessel in triplicate. For concentrated cell culture media, the volume of medium assayed was normalized to equivalent amounts of cells used for conditioning the media, as determined by the lysate protein concentration. Initial rates of fluorescent product formation were obtained from the slope of continuous readings taken over 30 minutes following the addition of substrate. Initial reaction rates were linear with both time and protein concentration (see Results).

RTPCR and antisense experiments. Quantitative realtime reverse transcriptase polymerase chain reaction was performed by standard protcols in the Genomics Technology Support Facility, Michigan State University. Primer sequences were designed on the basis of

published sequence information and the public domain software Primer3 (MIT, Cambridge, MA). The annealing temperatures for PCR reactions were optimized for each primer by preliminary trials. The identity of expressed genes was determined by expected size of the PCR product in 1.6% agarose gels, followed by excision and sequencing of the PCR product.

For RTPCR of rat CatD, the primers used were: (primer set 1) coding = 5'-ACACTGTGTCGGTTCCATGT-3', and uncoding = 5'-TGCGATGAATACGACTCC-AG-3', which produces a PCR product of 101bp, and (primer set 2) coding = 5'-GCGTCTTGCTGCTCATTCTC-3', and uncoding = 5'-TGGGACCTTTAAGGATCA-GG-3', which produces a PCR product of 141bp.

For antisense studies, phosphorothioated control and antisense oligonucleotides against CatD (22-mers) were designed through published sequence information and public domain software, synthesized and transfected into primary rat AEC (both at 40nM final concentration) using the lipofectin reagent OligofectAMINE (Invitrogen Life Technologies, Grand Island, NY) at 4ul/ml as the vehicle, diluted in the OPTIMEM medium accompanying the lipofectin. The control nucleotides were of the same length and base composition as the antisense, but with scrambled sequence. The oligonucleotide:lipofectin ratio was optimized to yield transfection efficiencies of 50-75% with minimal cell loss or detachment. Transfection efficiency was monitored with FITC-labeled 25-mer oligonucleotide for luciferase (not shown). Transfections were conducted for 4 hours followed by 5 times washing with serum-free cell culture medium, as described earlier (14,15). Immediately thereafter, BLEO or vehicle was applied as indicated for 20 hours. The transfection protocol itself had no significant effect on basal or BLEO-induced apoptosis (see Results). Phosphorothioated oligonucleotide sequences were: (CatD antisense) 5’-

CATATAGTTTTGCTTCTGTCCT-3', and (CatD scramble) 5'-TGCCCTATATGTTAGTTC-TTTC-3'.

RESULTS

Measurements of cathepsin D (CatD) enzymatic activity with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 revealed CatD enzymatic activity in lysates of purified rat alveolar epithelial cells (AECs, Figure 1); the generation of fluorescent product was linear with time and lysate protein concentration. Incubation of primary AEC cultures with bleomycin for 20 hours, at a concentration previously shown to stimulate AEC apoptosis (25mU/ml, 8), significantly increased the activity of CatD in both AEC lysates and in the serum-free cell culture medium (Figure 2). The aspartyl protease inhibitor pepstatin A (pepA) inhibited the CatD activity by over 90%.

Apoptosis inducers are known to increase CatD activity and mRNA in other cell types (5,18). Analyses of CatD mRNA in primary AECs by RTPCR (Figure 3A) revealed PCR products of the correct size expected from two different primer sets, but no apparent change in response to bleomycin. Sequencing of both PCR products verified the specificity of the PCR for rat CatD (not shown). Quantitative analyses of CatD mRNA by realtime PCR was unable to detect significant changes in the mRNA in response to bleomycin challenge (Figure 3B). In contrast, western blotting of AEC lysates and culture media with CatD-specific antibodies and high resolution gels (Figure 3) revealed bleomycin-induced increases in immunoreactive CatD proteins in the cell culture medium, but apparently not in the cell lysates. Bleomycin increased isoforms of CatD of apparent MW 52, 48 and 44kdal in the culture medium, whereas a 44kdal protein was the major immunoreactive isoform of CatD present in AEC lysates.

To begin determining if CatD might play a role in apoptosis of AECs as it does in other cell types, AEC apoptosis was evaluated in the presence and absence of the aspartyl protease inhibitor pepstatin A. In Figure 5, pepstatin A (pepA) inhibited bleomycin-induced nuclear

fragmentation of primary AEC by 76% ($p<0.01$) and reduced bleomycin-stimulated caspase 3 activity by 47% ($p<0.05$). The pepstatin A alone did not affect basal nuclear fragmentation or caspase 3 activity.

As an alternate test of the role of CatD in AEC apoptosis, phosphorothioated antisense oligonucleotides specific for CatD mRNA were designed and transfected transiently into primary rat AEC with lipofectin (LIPO, Figure 6). In Figure 6A, the antisense oligonucleotides (AS) significantly reduced the immunoreactive CatD released into AEC culture media, as determined by western blotting on low resolution gels. In contrast, scrambled-sequence control oligonucleotides (SCR), of the same length and base composition as the antisense, did not reduce CatD immunoreactivity. In Figure 6B, pretreatment of primary AECs with the same antisense oligonucleotides used in panel A (AS) reduced bleomycin-induced nuclear fragmentation by 48% ($p<0.05$).

Recent work from this laboratory showed that the induction of AEC apoptosis by bleomycin requires *de novo* synthesis of angiotensin II (ANGII) and its subsequent binding to ANG receptor subtype AT1 (8). To begin addressing the theory that the primary role of CatD in AEC apoptosis is its ability to process angiotensinogen to the peptide angiotensin I, CatD knockdown strategies were evaluated for the ability to prevent AEC apoptosis in response to a synthetic angiotensinogen fragment consisting of amino acids 1-14. This domain of angiotensinogen contains the catalytic sites for both CatD and angiotensin converting enzyme (ACE), which together generate angiotensin II. To confirm this premise with the reagents currently commercially available for this study, the purified angiotensinogen fragment 1-14 (F1-14) was treated in vitro (without cells) with purified ACE or purified CatD enzymes (Figure 7A). As expected, treatment of F1-14 with both ACE and CatD together resulted in significant

production of the peptide ANGII (Figure 7A), as measured by an ELISA that detects ANGII but not ANGI or angiotensinogen. In contrast, neither purified ACE alone nor purified CatD alone could convert F1-14 to the peptide ANGII. In Figure 7B, incubation of primary rat AECs with fragment F1-14 alone in serum-free culture medium (but without added enzymes) yielded significant production of ANGII, confirming the constitutive expression of ANG converting enzymes by primary AEC (16).

In Figure 8, incubation of primary rat AEC with F1-14 induced apoptosis detected by nuclear fragmentation. The apoptosis was completely blocked by the nonspecific or AT1-selective ANG receptor antagonists saralasin (SARAL) or L158809, respectively. Moreover, the apoptosis was inhibited 96% by the CatD inhibitor pepstatin A (pepA), and was reduced 77% by neutralizing antibodies specific for CatD (CatD AB, both $p<0.001$).

DISCUSSION

A role for the aspartyl protease CatD in apoptosis has been shown previously in HeLa cells exposed to interferon-γ, Fas ligand or TNF-α (1) and in PA1 ovarian cancer cells (18). The activity of CatD is upregulated by the apoptosis inducer adriamycin in PA1 cells and in ML1 leukemia cells and U1752 lung cancer cells (18). Although the aspartyl protease inhibitor pepstatin A could block apoptosis in these cell types, the exact mechanism(s) by which CatD participates in the execution of apoptosis is unclear. In accord with the known ubiquitous expression of CatD as a lysosomal protease (10), it has been suggested that this and other lysosomal proteases might be involved in the production of a bioactive molecule required for apoptosis of PC12 cells in response to trophic withdrawal (5).

Cathepsin D also is known to be one of the enzymes capable of proteolytically processing the liver-derived and serum-borne protein angiotensinogen to the peptide angiotensin I, a function normally performed in the serum by the kidney-derived enzyme renin (2). On the other hand, evidence from several nonpulmonary cell types has established CatD as the primary enzyme that converts angiotensinogen to ANGI within local “intrinsic” angiotensin systems, independently of renin (2,17).

Recent studies from this laboratory have shown that bleomycin-induced apoptosis of alveolar epithelial cells requires the autocrine synthesis of angiotensinogen, angiotensin II and its binding to ANG receptor AT1 (8). Those data were consistent with related studies showing that purified ANGII itself was a potent inducer of apoptosis in AEC (16), and implied that AEC express enzymes capable of converting angiotensinogen to ANGII. Although the same study showed constitutive expression of angiotensin converting enzyme (ACE) by alveolar epithelial

cells, the aspartyl protease required for providing the substrate for ACE (ANGI) in AEC was unknown.

The data herein strongly suggest that CatD functions in this capacity in AEC; bleomycin-induced nuclear fragmentation and caspase 3 activity were significantly reduced by the aspartyl protease inhibitor pepstatin A (Figure 5) or by antisense oligonucleotides against CatD mRNA (Figure 6). In earlier investigations, bleomycin-induced apoptosis of AEC was competely blocked by specific angiotensin receptor antagonists or ANG-neutralizing antibodies (8); this finding lead to the theory that autocrine generation of ANGII is required for AEC apoptosis regardless of the initiating stimulus (11). In the light of those results, the finding that CatD antisense treatment did not completely block bleomycin-induced nuclear fragmentation (48%, Figure 6B) might indicate a potential role for additional protease(s) in angiotensinogen processing and subsequent AEC apoptosis. This interpretation is consistent with the finding that the protease inhibitor pepstatin A, which blocks all aspartyl proteases, also was incapable of complete blockade of nuclear fragmentation (76%, Figure 5) despite complete inhibition of CatD enzyme activity in AEC lysates (Figure 2). On the other hand, the antisense treatment, which is at least theoretically specific, did not completely eliminate immunoreactive CatD detected by western blotting (Figure 6A). Thus, it is difficult to know with certainty if the incomplete blockage of apoptosis is due to inefficient CatD knockdown or additional proteases activities.

Regardless, studies of angiotensinogen fragment 1-14 (Figures 7&8) are consistent with the theory that CatD is required for the conversion of angiotensinogen to ANGII by AEC, and with earlier work. For example, the finding that incubation of primary rat AECs with the fragment F1-14 alone in serum-free culture medium (but without added enzymes) yielded significant production of ANGII (Figure 7) is consistent with the earlier demonstration of

constitutive, albeit low, expression of both ACE and an unidentified aspartyl protease by primary AECs (16). Moreover, the complete abrogation of AEC apoptosis in response to angiotensinogen fragment F1-14 by the nonselective and AT1-selective ANG receptor antagonists saralasin and losartan (Figure 8) confirmed that the induction of apoptosis was dependent on both the generation of ANGII from F1-14 and the binding of ANGII to receptor AT1. Those results also are consistent with our earlier demonstrations that AT1 receptor mediates AEC apoptosis in response to bleomycin (8), amiodarone (3,13) or purified ANGII (9). Most important, the findings that AEC apoptosis in response to F1-14 was essentially abrogated by either pepstatin A or by CatD antibodies (Figure 8) strongly suggest that the conversion of angiotensinogen to ANGI, and subsequently ANGII to induce AEC apoptosis, is dependent on CatD activity.

The upregulation of CatD activity by bleomycin in this study is consistent with the earlier findings that CatD is upregulated in alveolar epithelial cells in fibrotic human lung (6) and is induced in the L132 lung cell line during apoptosis in vitro (7). In other cell types, apoptosis inducers upregulate both CatD protein and mRNA (18), which suggests control of activation at the level of RNA. In contrast, RTPCR studies of AEC transcripts after bleomycin treatment failed to detect changes in CatD mRNA (Figure 3) despite significant increases in CatD activity (Figure 2) and immunoreactive protein by western blotting (Figure 4). It is possible that the relatively few sampling times chosen for realtime analyses of CatD mRNA may have missed a transient but shortlived increase in the mRNA that might be revealed by a more exhaustive timecourse study. On the other hand, CatD is known to undergo activation by proteolytic mechanisms as well; in human U937 cells, CatD was shown to undergo processing of the inactive prepro- isoform (52kdal) to the active proCatD (48kdal) and an active 32kdal isoform, in

response to autocatalysis of the enzyme induced by the direct binding of the apoptosis mediator ceramide (4).

Consistent with those findings, western blotting of rat AEC lysates did reveal bleomycin-induced increases in several isoforms of apparent MW 44-52kdal. However, two of the isoforms shown to be increased in AEC media (52 and 48kdal, see “medium” in Figure 4) are larger than the primary isoform detected intracellularly in AEC (44kdal, “monolayer” in Figure 4). This finding argues against proteolytic processing alone as a mechanism of CatD activation in AEC. Thus, the exact mechanism(s) by which bleomycin upregulates CatD in AEC is unknown, but will pose an interesting problem for future studies. Pepstatin A-inhibitable CatD activity also was upregulated by amiodarone (13) and TNF-alpha (14), both of which induce apoptosis in AECs (13,14), but a determination of whether the requirement for CatD is universal to all proapoptotic stimuli for AEC will require further investigation.

In summary, bleomycin upregulated CatD enzymatic activity and immunoreactive protein in primary cultures of rat alveolar epithelial cells (AEC). Apoptosis of cultured AEC in response to bleomycin was significantly inhibited by the aspartyl protease inhibitor pepstatin A or by antisense oligonucleotides against CatD mRNA. The same inhibitors also prevented the enzymatic processing of a synthetic fragment of angiotensinogen (amino acids 1-14), and completely blocked AEC apoptosis in response to the same peptide. These data are consistent with earlier studies showing that apoptosis of AEC in response to bleomycin requires the autocrine synthesis and proteolytic processing of angiotensinogen to angiotensin II, and suggest that the proteolytic processing requires CatD. The data herein also suggest that blockade of CatD and other aspartyl proteases might provide a potential strategy for preventing AEC apoptosis and lung injuries that involve this mode of cell death.

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Figure 1. Cleavage of a fluorogenic substrate for Cathepsin D (CatD) is dependent on time and protein concentration. Lysates of primary alveolar epithelial cells (AECs) were incubated with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2, and generation of fluorescent product was monitored continuously over 30 minutes (see Methods for details). Note linearity of product formation with time and amount of AEC lysate.

Figure 2. Bleomycin upregulates CatD activity and release from AECs. Primary cultures of rat alveolar epithelial cells (AECs) were exposed to bleomycin (BLEO) for 20 hours at a concentration known to induce AEC apoptosis (25mU/ml, 8). CatD activity was measured in cell lysates as described in Figure 1, in the presence or absence of the aspartyl protease inhibitor pepstatin A (pepA).

Inset: CatD activity was measured in concentrated cell culture medium collected from BLEO-treated (BL) and untreated (C) cells studied in panel A. Bars are the mean + S.E.M. of n = 6; * = $p<0.01$ versus untreated control (CTL) by ANOVA and Student-Newman-Keul's test.

Figure 3. Bleomycin does not alter steady-state levels of CatD mRNA. A: PCR products from two different primer sets (1 and 2, see Methods) used to amplify CatD mRNA by RTPCR; starting material was total RNA isolated from primary rat AECs exposed to BLEO or vehicle for the indicated times. B: Realtime RTPCR of CatD mRNA (primer set 2) at the indicated times after exposure to BLEO (see Methods). B-MG = β-microglobulin; bars are the mean+S.E.M. of three separate AEC cultures.

Figure 4. Bleomycin increases the release of immunoreactive CatD protein from cultured AECs. Primary cultures of AECs were exposed to bleomycin (BLEO) as in Figure 2; detergent lysates were harvested from the cells (monolayer), and the cell culture medium was collected and concentrated. Equal amounts of lysate protein (10ug/lane) or volume of culture medium (equivalent to 10^5 cells) were subjected to western blotting with Cat-D-specific antibodies (see Methods). Note increases in immunoreactive proteins of apparent MW~ 52, 48 and 44kdal (arrowheads) in medium from BLEO-treated AECs.

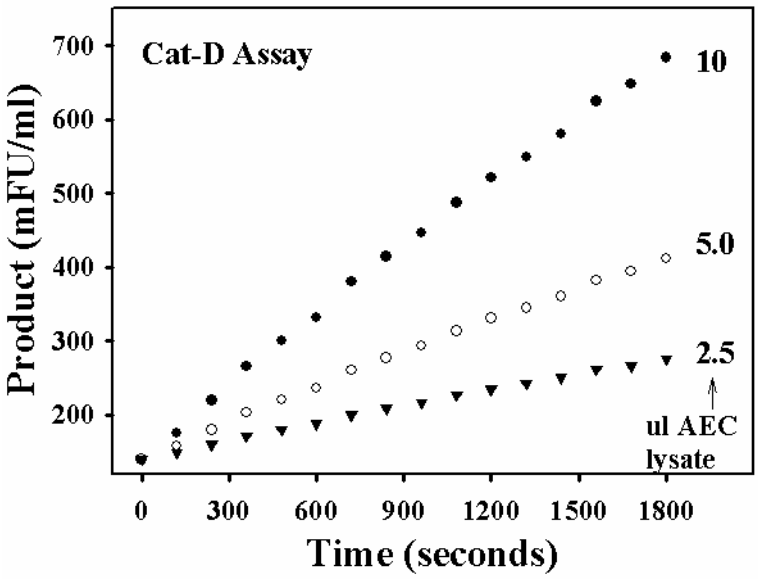
Figure 5. Pepstatin A inhibits bleomycin-induced apoptosis of AECs in vitro. Primary cultures of rat AECs were exposed to BLEO in the presence or absence of pepstatin A (pepA) at 100uM (1,18). Apoptosis was quantitated by scoring of nuclear fragmentation (14,15) with propidium iodide (panel A) or by the enzymatic activity of Caspase 3 (B). See Methods for details. Bars are the mean + S.E.M. of n = 6; * = $p<0.01$ versus untreated control and ** = $p<0.05$ versus BLEO by ANOVA and Student-Newman-Keul's test.

Figure 6. Antisense oligonucleotides reduce CatD immunoreactivity and inhibit bleomycin-induced apoptosis of AECs in vitro. A: Antisense (AS) or scrambled-sequence oligonucleotides (SCR) were transfected into primary cultures of rat AECs in the presence of lipofectin (LIPO, see Methods), without challenge with bleomycin (CTL). Western blotting of concentrated cell culture media was performed with CatD-specific antibodies; note decrease in immunoreactive CatD by AS but not SCR oligonucleotides. B: After antisense oligonucleotide transfection as in panel A, AECs were challenged with BLEO (25mU/ml) and harvested for detection of

fragmented nuclei as in Figure 5. Bars are the mean + S.E.M. of n = 3; * = $p<0.01$ versus untreated control (CTL) and ** = $p<0.05$ versus BLEO by ANOVA and Student-Newman-Keul's test.

Figure 7. Production of ANGII from angiotensinogen fragment 1-14 in vitro.
A: Angiotensinogen fragment 1-14 (F1-14, 5uM) was incubated in vitro (without cells) with the indicated purified enzymes; ANGII was measured in the reaction buffer by specific ELISA (see Methods for details). Note production of ANGII by the combination of purified CatD + purified angiotensin converting enzyme (ACE), but not by either enzyme alone. B: Primary cultures of AECs were exposed to 5uM F1-14, and ANGII was measured in the serum-free cell culture medium; note production of ANGII by AECs challenged with F1-14, but not by untreated AECs.

Figure 8. CatD-dependent induction of AEC apoptosis by angiotensinogen fragment 1-14.
Primary cultures of AEC were incubated with F1-14 as in Figure 7, in the presence or absence of pepstatin A (pepA, 1uM); CatD-specific neutralizing antibodies (CatD AB, 1:100) and the ANG receptor antagonists saralasin (SARAL, 50ug/ml) or L158809
(10^{-6} M). See Methods for details. Bars are the mean + S.E.M. of n = 3; * = $p<0.001$ versus untreated control (CTL) and ** = $p<0.001$ versus F1-14 by ANOVA and Student-Newman-Keul's test.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Product (mFU/ml)
700
600
500
400
300
200
0
300
600
900
1200
1500
1800
Time (seconds)
10
5.0
2.5
ul AEC
lysate

Figure 1

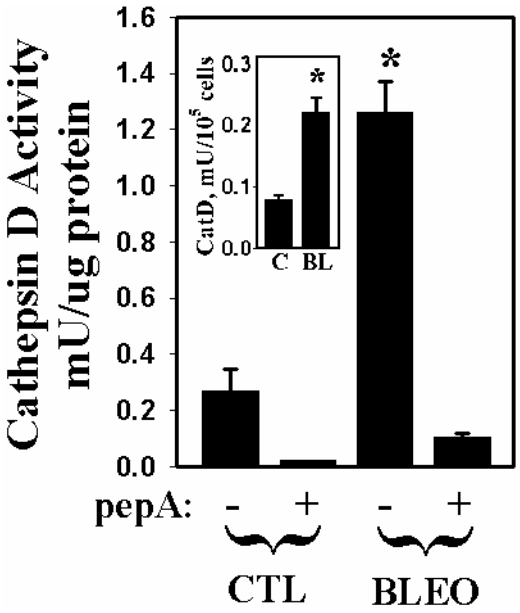
Cathepsin D Activity
mU/ug protein
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CatD, mU/10^5 cells
0.3
0.2
0.1
0.0
C
BL
*
*
pepA:
-
+
-
+
CTL
BLEO

Figure 2

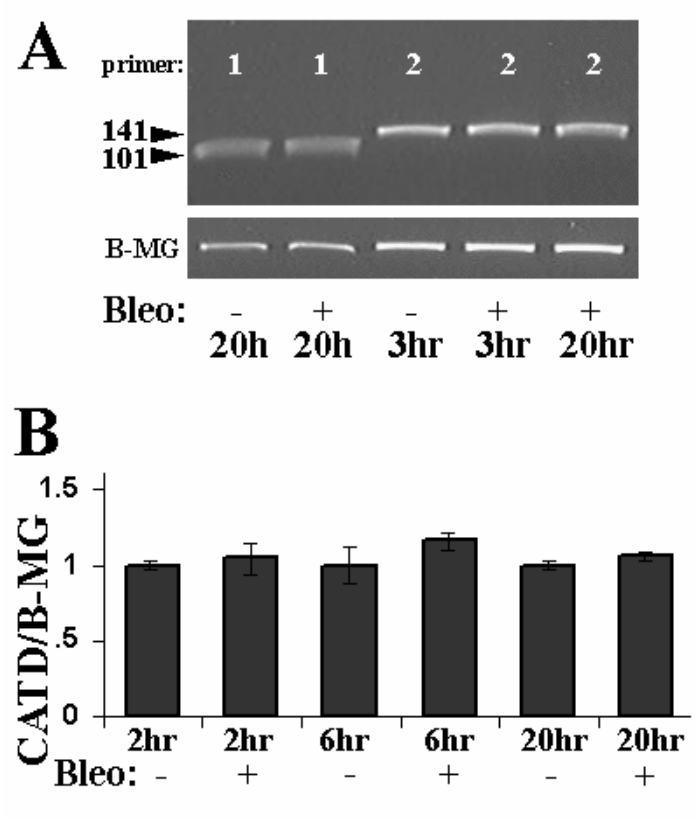
A
primer:
1
1
2
2
2
141
101
B-MG
Bleo:
-
+
-
+
+
20h
20h
3hr
3hr
20hr
B
CATD/B-MG
1.5
1
.5
0
2hr
2hr
6hr
6hr
20hr
20hr
Bleo:
-
+
-
+
-
+

Figure 3

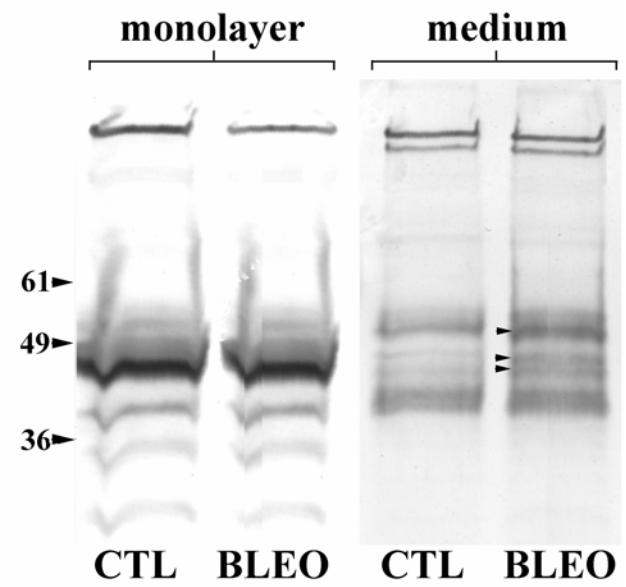

Figure 4

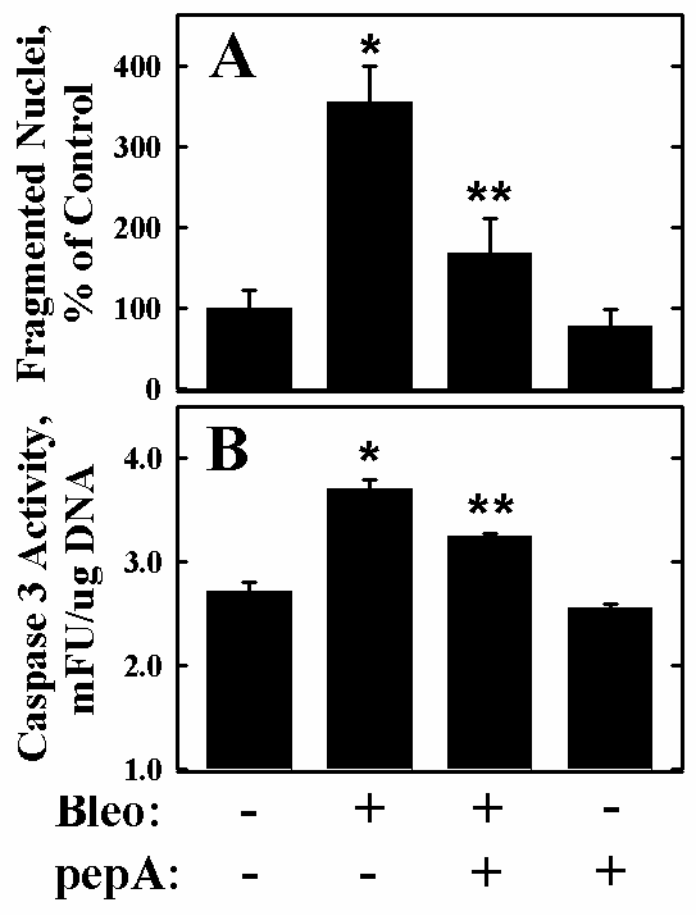
A
B
Fragmented Nuclei, % of Control
Caspase 3 Activity, mFU/ug DNA
400
300
200
100
0
4.0
3.0
2.0
1.0
*
**
Bleo: - + + -
pepA: - - + +

Figure 5

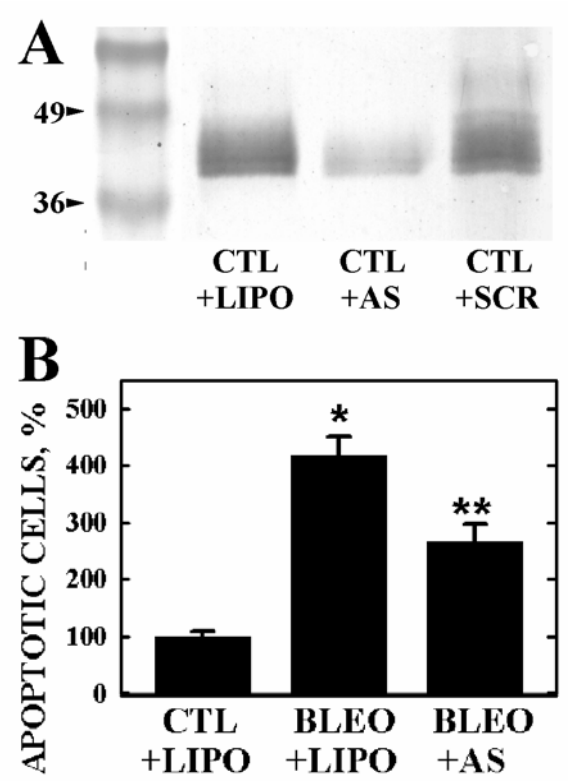
A
49
36
CTL +LIPO
CTL +AS
CTL +SCR
B
APOPTOTIC CELLS, %
500
400
300
200
100
0
*
**
CTL +LIPO
BLEO +LIPO
BLEO +AS

Figure 6

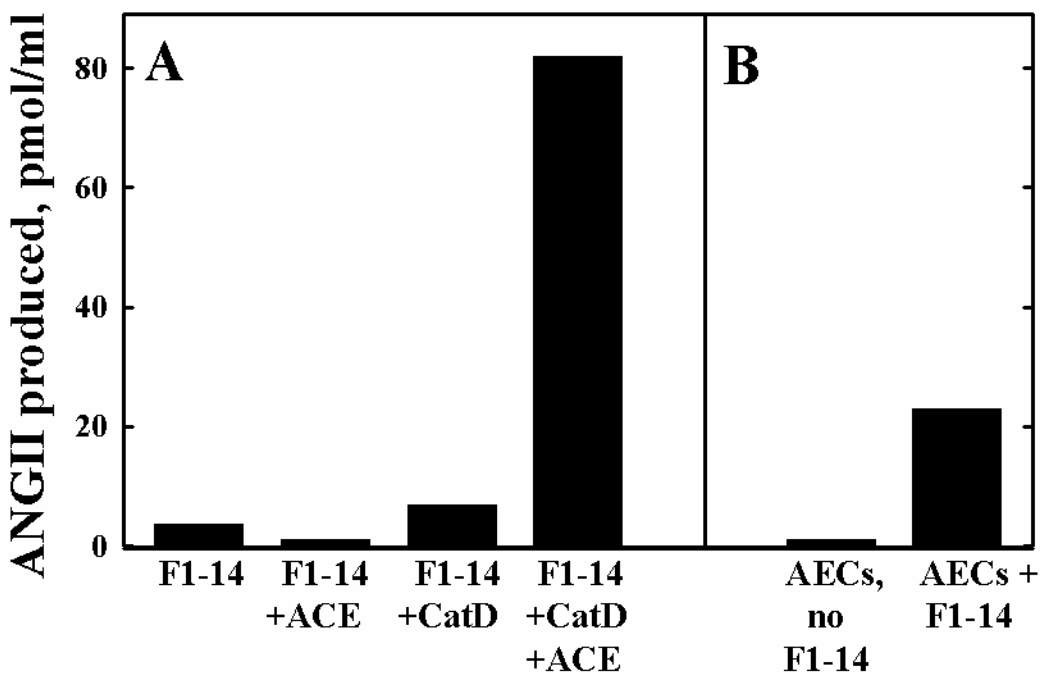
ANGII produced, pmol/ml
0
20
40
60
80
A
B
F1-14
F1-14 +ACE
F1-14 +CatD
F1-14 +CatD +ACE
AECs, no F1-14
AECs + F1-14

Figure 7

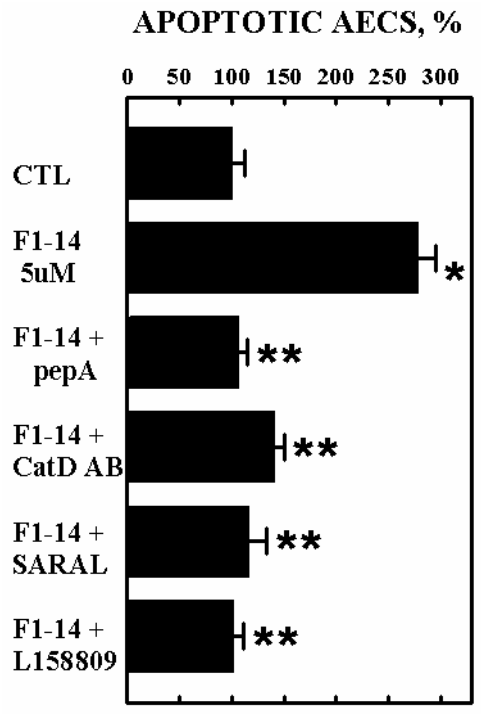
APOPTOTIC AECS, %
0
50
100
150
200
250
300
CTL
F1-14
5uM
*
F1-14 +
pepA
**
F1-14 +
CatD AB
**
F1-14 +
SARAL
**
F1-14 +
L158809
**

Figure 8