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Abrogation of ER stress-induced apoptosis of alveolar epithelial cells by angiotensin 1–7

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Uhal BD, Nguyen H, Dang M, Gopallawa I, Jiang J, Dang V, Ono S, Morimoto K. Abrogation of ER stress-induced apoptosis of alveolar epithelial cells by angiotensin 1–7. Am J Physiol Lung Cell Mol Physiol 305: L33–L41, 2013. First published April 26, 2013; doi:10.1152/ajplung.00001.2013.—Earlier work showed that apoptosis of alveolar epithelial cells (AECs) in response to endogenous or xenobiotic factors is regulated by autocrine generation of angiotensin (ANG) II and its counterregulatory peptide ANG1–7. Mutations in surfactant protein C (SP-C) induce endoplasmic reticulum (ER) stress and apoptosis in AECs and cause lung fibrosis. This study tested the hypothesis that ER stress-induced apoptosis of AECs might also be regulated by the autocrine ANGII/ANG1–7 system of AECs. ER stress was induced in A549 cells or primary cultures of human AECs with the proteasome inhibitor MG132 or the SP-C BRICHOS domain mutant G100S. ER stress activated the ANGII-generating enzyme cathepsin D and simultaneously decreased the ANGII-degrading enzyme ACE-2, which normally generates the antiapoptotic peptide ANG1–7. TAPI-2, an inhibitor of ADAM17/TACE, significantly reduced both the activation of cathepsin D and the loss of ACE-2. Apoptosis of AECs induced by ER stress was measured by assays of mitochondrial function, JNK activation, caspase activation, and nuclear fragmentation. Apoptosis induced by either MG132 or the SP-C BRICHOS mutant G100S was significantly inhibited by the ANG receptor blocker saralasin and was completely abrogated by ANG1–7. Inhibition by ANG1–7 was blocked by the specific mas antagonist A779. These data show that ER stress-induced apoptosis is mediated by the autocrine ANGII/ANG1–7 system in human AECs and demonstrate effective blockade of SP-C mutation-induced apoptosis by ANG1–7. They also suggest that therapeutic strategies aimed at administering ANG1–7 or stimulating ACE-2 may hold potential for the management of ER stress-induced fibrotic lung disorders.

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by a synthetic proteasome inhibitor (MG132) or by an SP-C BRICHOS domain mutant (G100S) causes proapoptotic alterations of the autocrine ANGII/ANG1–7 system and, furthermore, can be prevented by either ANG receptor blockade or by the antiapoptotic peptide ANG1–7.

**MATERIALS AND METHODS**

**Reagents and materials.** The ANG receptor nonselective saralasin, propidium iodide, and clasto-lactacystin β-lactone (CLBL) were obtained from Sigma Chemical, St. Louis, MO. Angiotensin 1–7, ANG1–7 (α-Ala7) and A779 (α-Ala7-Ang1–7), and MG132 (carboxybenzoxyl-Leu-Leu-uncinal) were obtained from GenScript USA, Piscataway, NJ. The cathepsin D fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-ο-Arg-NH2 was obtained from Peptides International, Louisville, KY. 3,3’-dihexyloxacarbocyanine iodide (DiOC6) was obtained from Molecular Probes, Eugene, OR. Antibodies for the detection of phospho-JNK, total JNK, ACE-2, the active forms of caspase-7, caspase-8, cytochrome c, and β-actin were all from Cell Signaling, Danvers, MA. N-(R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-t-butyralanyl-alaninyl-l-alanine, 2-aminooethyhl amide (TAPI-2) was obtained from Calbiochem, Billerica, MA. All other materials were of reagent grade and were obtained from Sigma Chemical.

**Cell culture.** The A549 human type II epithelial cell-derived cell line A549 was obtained from ATCC (Manassas, VA) and was cultured in F12 medium with 10% serum. Primary human alveolar epithelial cells were purchased from ScienCell Research Laboratories (Carlsbad, CA) and were cultured in specific media formulated by that supplier. The mouse lung epithelial cell line MLE-12, a kind gift from the laboratory of Dr. Jeffrey Whitsett, University of Cincinnati, was grown in complete HITES medium. The primary cells were studied at day 2 of culture, a time at which they are type II cell-like by accepted morphological and biochemical criteria (22). All cells were grown in 24- or 6-well chambers and were analyzed at subconfluent densities of 50–80% except where indicated. All subsequent incubations with ANG1–7 and/or other test agents were performed in serum-free medium unless otherwise indicated. In all studies, cells were exposed to inhibitors or antagonists 30 min before exposure to MG132 or SP-C plasmids for 5 min to 30 h as indicated. For extended exposures to A779 and ANG1–7 (Figs. 6–8), cells were exposed to test agents as just described, and after 1 h culture media were replaced with new media containing fresh A779, ANG1–7, and/or MG132. The replacement of A779 and ANG1–7 were continued every 3 h thereafter until cell harvesting to compensate for the low biological half-lives of these peptides (data not shown).

**G100S mutant and wild-type SP-C plasmids.** The DNA sequences for human wild-type and G100S mutant SP-C carried in the pIREs dsRED plasmid were constructed in the Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki Japan (17). The G100S- and wild-type-containing plasmids were amplified using the Plasmid Plus Maxi Kit (Qiagen, Valencia CA). The manufacturer’s protocol was modified to obtain the highest yield of plasmid DNA possible. The wild-type and mutant SP-C sequences were verified by sequencing at the Genomics Core at the Research Technology Support Facility at Michigan State University by using the forward primer 5’-GACCTTCCAATAATGCTGAACTCCT-3’ and reverse primer 5’- AACCGGCCCTGCGCCAGTACGTTA3’ (17).

**Transfection protocol.** A549 cells were seeded into 24-well plates to a density of 75% confluence in F12 medium + 10% serum. After 24 h, the cells were serum starved for 24 h before transfection. The cells were transfected at a ratio of 0.50 μg plasmid DNA to 1.875 μL Lipofectamine 2000 (Invitrogen Life Technologies, Grand Island, NY), and 50 μL of the transfection solution was added to each well in a dropwise manner. The cells were incubated at 37°C with 5% CO2; after 4 h, the medium with the transfection solution was removed and replaced with 500 μL of serum-free medium. At this time, 5 μL of a stock solution of saralasin or ANG1–7 and/or A779 was added to the desired wells for a final concentration of 50 μg/mL and 1 × 10–7 M, respectively. Cells were placed back in the incubator. Every 3 h, ANG1–7 and A779 were replaced at the same final concentration as mentioned above. At 28 h, the plates were removed from the incubator and assayed for nuclear fragmentation.

**Nuclear fragmentation assay.** Detection of apoptotic cells by nuclear fragmentation with propidium iodide (PI) was conducted as described earlier (13) after enzymatic digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 μg/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, equating fragmented nuclei with apoptosis was verified by in situ end labeling of fragmented DNA (12, data not shown). Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group. The active forms of caspase-7 and caspase-8 and cytosolic cytochrome c were detected by Western blotting using antibodies specific for the active (cleaved) forms.

**Western blotting.** Cells were lysed either in an Nonidet P-40-based lysis buffer containing protease inhibitors (for ACE-2, cathepsin D, cytochrome c, or caspase quantitations) or, for detection of phosphoproteins, with a modified lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 μM EGTA, 1.5 mM MgCl2, 100 μM sodium orthovanadate, and the protease inhibitor cocktail (Complete Mini, Roche, Nutley, NJ). Proteins were run on polyacrylamide gels and transferred to PVDF membranes. Immunoreactive bands were visualized by ECL detection systems (Thermo Scientific, Rockford, IL).

![Fig. 1.](http://ajplung.physiology.org/)
Student-Newman-Keuls post hoc test.

the assay chamber (see MATERIALS AND METHODS for details). Bars are means of 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 min following the addition of substrate. Initial reaction rates were linear with both time and protein concentration.

Assay of cathepsin D 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 (12). Briefly, aliquots of AEC lysates or concentrated cell culture medium were incubated in opaque 96-well culture plates (suitable for top reading in a fluorescence plate reader) in 1.0 M sodium acetate buffer, pH 4.0 containing 50 μM fluorogenic substrate. The total volume of reaction buffer, including sample, was 100 μL. 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 min following the addition of substrate. Initial reaction rates were linear with both time and protein concentration.

Estimation of mitochondrial membrane potential. The mitochondrial membrane potential (ΔΨ, MMP) in A549 cells was estimated with the lipophilic probe DiOC6 applied in PBS to A549 cells on 24-well culture vessels at a final concentration of 50 nM. After treatment with MG132 (10 μM) for 6 h in the presence or absence of ANG1–7 or saralasin, the cells were washed once and incubated with PBS containing 50 nM DiOC6 for 15 min at 37°C followed by assay in a fluorescence plate reader (BioTek, Winooski, VT) at 360 nm excitation and 420 nm emission. After data acquisition, the cells were fixed with 70% ethanol, incubated with 10 μM Hoechst 33342 in PBS for 30 min more, and then reanalyzed at the same wavelengths for quantitation of total cellular DNA. DiOC6 fluorescence data were then normalized to the total DNA in each culture well.

RESULTS

In agreement with the results of others (7, 17), the ER stress marker BiP (GRP78) was upregulated in the human AEC cell line A549 by the synthetic proteasome inhibitor MG132 (Fig. 1A) or by the SP-C BRICHOS domain mutant G100S (Fig. 1B), one of the SP-C mutations that causes lung fibrosis secondary to epithelial ER stress and the UPR (17). In agreement with the work of Maguire and colleagues (15) using a different SP-C mutant (Δ-exon4), the transcription factor CHOP was upregulated by MG132 (Fig. 1A) but not by the G100S mutant of SP-C (Fig. 1B), suggesting that it also acts in a CHOP-independent manner to induce apoptosis.

In the light of earlier works showing that the autocrine angiotensin (ANG)/ANG1–7 systems regulate bleomycin-induced apoptosis of AECs (13, 23), the effects of ER stress on key components of the autocrine ANGII/ANG1–7 system were tested in A549 cells challenged with MG132. At the same concentration of MG132 that induced BiP and CHOP expression in Fig. 1A549 by the synthetic proteasome inhibitor MG132 (Fig. 1A) or by the SP-C BRICHOS domain mutant G100S (Fig. 1B), one of the SP-C mutations that causes lung fibrosis secondary to epithelial ER stress and the UPR (17). In agreement with the work of Maguire and colleagues (15) using a different SP-C mutant (Δ-exon4), the transcription factor CHOP was upregulated by MG132 (Fig. 1A) but not by the G100S mutant of SP-C (Fig. 1B), suggesting that it also acts in a CHOP-independent manner to induce apoptosis.

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Cathepsin D is required for AEC apoptosis in response to bleomycin through its cleavage of angiotensigen (12); in Fig. 2B, complete inhibition of cathepsin D enzymatic activity by pepstatin A supports the specificity of the enzyme assay for aspartyl protease induction by MG132. Figure 3 shows that ER stress induced by MG132 also downregulated the ANGII-degrading enzyme ACE-2, beginning at a concentration of 7.5 μM. ACE-2 was also downregulated by the alternate proteasome inhibitor CLBL if applied to A549 cells cultured at a higher cell density, suggesting that the downregulation was due to proteasome inhibition rather than density-dependent effects on cell proliferation (21). In agreement with that interpretation, the G100S BRICHOS domain mutant of SP-C, when transfected into A549 cells alongside wild-type SP-C in the same plasmid, also reduced ACE-2, especially the low molecular weight (MW) active form of the enzyme.

Previous work showed that ANGII is proapoptotic for AECs, but the product of its degradation by ACE-2 (i.e., ANG1–7) is antiapoptotic (23). Therefore, to begin testing whether changes in either ANGII or ANG1–7 might mediate ER stress-induced apoptosis of AECs, caspase activation by MG132 was examined in the presence or absence of the nonselective ANGII receptor antagonist saralasin or, separately, synthetic ANG1–7. As shown in Fig. 4, both saralasin and ANG1–7 significantly inhibited caspase 7 activation by MG132 (Fig. 4A), and synthetic ANG1–7 completely inhibited caspase 8 activation, both in A549 cells. Inhibition of caspase 8 by saralasin was less potent.

In accord with earlier reports that ER stress-induced apoptosis of AECs involves mitochondrial dysfunction leading to apoptosis signaling (15), the ability of saralasin or ANG1–7 to affect mitochondrial function was assessed in A549 cells challenged with MG132. In Fig. 5, either saralasin or ANG1–7 strongly inhibited MG132-induced cytochrome c release into the cytosolic fraction of A549 cells (Fig. 5A). Moreover, MG132 caused a reduction in the mitochondrial membrane potential (MMP, Fig. 5B) of A549 cells; the reduction in MMP was significantly inhibited or completely abrogated, respectively, by saralasin or by synthetic ANG1–7.

Earlier work demonstrated that the ability of ANG1–7 to block bleomycin-induced apoptosis of AECs was related to its ability to reduce JNK phosphorylation (23). Therefore, the effect of ANG1–7 or saralasin on JNK phosphorylation was examined in AECs challenged with MG132. Figure 6 shows complete blockade of MG132-induced JNK phosphorylation by ANG1–7 and partial blockade by saralasin, results similar to those of Fig. 5B for the mitochondrial membrane potential assay.

To determine whether ANG receptor blockade or ANG1–7 could inhibit functional markers of AEC death as well as the apoptosis signaling pathways just described, both A549 cells and primary cultures of human alveolar epithelial cells were challenged with MG132 and examined by nuclear fragmentation assay (13, 23), a measure of the final stages of apoptosis. As shown in Fig. 7, saralasin substantially inhibited and ANG1–7 completely inhibited MG132-induced nuclear fragmentation in either A549 cells (Fig. 7A) or in primary cultures of human lung alveolar epithelial cells (Fig. 7B). Moreover, the inclusion of A779 (Fig. 7B), a specific peptide antagonist of the ANG1–7 receptor mas (23), blocked the ability of ANG1–7 to abrogate MG132-induced nuclear fragmentation in the primary cultures of human AECs.

In Fig. 8, ER stress in response to the G100S mutation in the BRICHOS domain of SP-C (17) was examined for the possibility that G100S-induced apoptosis might also be mediated by the autocrine ANGII/ANG1–7 system in AECs. Figure 8A shows similar levels of SP-C overexpression in A549 cells transfected with plasmids expressing either wild-type or mutant (G100S) SP-C. In Fig. 8B, either saralasin or synthetic ANG1–7 completely eliminated the G100S-induced increase in nuclear fragmentation of A549 cells. Inhibition by ANG1–7 was reversed by the peptide antagonist A779 (t-Ala’m-Ang1–7), indicating that the inhibition is mediated by the ANG1–7 receptor mas.
In initial studies to begin determining the mechanisms by which ER stress reduces ACE-2 and activates cathepsin D in AECs, it was hypothesized that the ACE-2 ectodomain shedding enzyme ADAM17/TACE (TNF-α-converting enzyme, 4) might play a role in the loss of ACE-2. Figure 9A shows that the ADAM17/TACE-specific inhibitor TAPI-2 reversed the CLBL-induced loss of the low MW form of ACE-2 and also increased the high MW isoform (arrowhead), but overlap of the bands precluded accurate densitometry. In Fig. 9B, however, densitometry of all three isoforms of ACE-2 showed that TAPI-2 could abrogate the ACE-2 loss induced by the G100S mutant of SP-C. In Fig. 9C, coinubcation with TAPI-2 also significantly reduced the enzymatically active (low MW) isoforms of cathepsin D in response to either CLBL or MG132, suggesting a role for ADAM17/TACE in cathepsin D activation as well as ACE-2 loss.

Figure 10 shows a summary of the roles of the autocrine ANGII/ANG1–7 system in ER stress-induced apoptosis of alveolar epithelial cells. ER stress activates the ANGII-producing enzyme cathepsin D and reduces the ANGII-degrading- but ANG1–7-producing enzyme ACE-2. Both these actions shift the balance from the normally antiapoptotic ANG1–7/mas axis toward the proapoptotic ANGII-producing axis, promoting AEC apoptosis. Consistent with this model, ER stress-induced apoptosis in response to either the synthetic proteasome inhibitor MG132 or the SP-C BRICHOS domain mutant G100S could be prevented by ANGII receptor blockade (saralasin) or, more completely, by exogenous ANG1–7. The inhibitory action of ANG1–7 is mediated by its receptor mas, which reduces JNK phosphorylation by mechanisms yet to be elucidated.

DISCUSSION

ER stress of alveolar epithelial cells in response to mutations in the BRICHOS domain of SP-C (9, 17) and in other proteins involved in the intracellular trafficking of surfactant components (27) are now believed to be the cause of lung fibrosis in the families in which these mutations were discovered. A key mechanism common to all these mutations is their ability to induce apoptosis of alveolar epithelial cells, the only cell type in which the SP-C mutations are expressed, secondary to the ER stress and the UPR to misfolded mutant SP-C (9).

The concept that induction of apoptosis specifically in alveolar epithelial cells is sufficient to induce a fibrogenic response in the lungs is supported by a vast body of evidence that began almost 40 years ago. Adamson and Bowden (1) and later Haschek and Witschi (6) were the first to suggest that the inability of the alveolar epithium to repair, rather than inflammation, was the key determinant of whether lung injury healed normally or progressed to fibrosis. Many years later, blockade of experimental lung fibrogenesis with caspase inhibitors (8, 25) or by deletion of genes critical to apoptosis signaling pathways (2) gave additional experimental support to
the angiotensin system, i.e., the ACE-2/ANG1–7/mas axis, also regulates AEC apoptosis in concert with the ANGII side of the system just discussed (23). In that study of bleomycin-induced AEC apoptosis, ACE-2 expressed constitutively by AECs was shown to be a survival factor through its abilities to 1) degrade ANGII and thereby limit its accumulation and 2) generate the ANGII degradation product ANG1–7, which potently inhibits AEC apoptosis by reducing JNK phosphorylation through the ANG1–7 receptor mas (23).

The results reported herein demonstrate that the autocrine ANGII/ANG1–7–mas axis also regulates AEC apoptosis in response to ER stress induced by either exogenous proteasome inhibition (MG132) or by a mutant in the BRICHOS domain of SP-C (G100S). In response to either inducer, the protective antiapoptotic peptide ANG1–7 could completely abrogate nuclear fragmentation in A549 cells or in primary cultures of human AECs (Fig. 7). This result suggests a more potent role for the ACE-2/ANG1–7/mas axis in ER stress-induced apoptosis, relative to the ANGII/ANG receptor axis studied earlier (13). Moreover, these results are particularly exciting in the light of recent pilot clinical trials of the AT1 receptor blocker losartan, which yielded promising positive results, albeit lim-

A more recent study demonstrated that the “alternate” side of the angiotensin system, i.e., the ACE-2/ANG1–7/mas axis, also regulates AEC apoptosis in response to ER stress induced by either exogenous proteasome inhibition (MG132) or by a mutant in the BRICHOS domain of SP-C (G100S). In response to either inducer, the protective antiapoptotic peptide ANG1–7 could completely abrogate nuclear fragmentation in A549 cells or in primary cultures of human AECs (Fig. 7). This result suggests a more potent role for the ACE-2/ANG1–7/mas axis in ER stress-induced apoptosis, relative to the ANGII/ANG receptor axis studied earlier (13). Moreover, these results are particularly exciting in the light of recent pilot clinical trials of the AT1 receptor blocker losartan, which yielded promising positive results, albeit lim-

those early theories. By analogy, the more recent discovery of mutations in a protein that is expressed only in AECs (SP-C), and that results in the apoptosis of this cell type and lung fibrosis, might be considered proof of the concept first put forth by the foresightful investigators of the mid-1970s.

Accordingly, an understanding of the factors that control the survival of AECs is key to understanding the initiation and progression of lung fibrogenesis and how it might be manipulated therapeutically. Previous work from this laboratory had demonstrated that apoptosis of AECs in response to bleomycin, Fas ligand, or TNF-α (13, 24, 26) is mediated by an autocrine angiotensin system that is initiated by increased transcription of the AGT gene in the AEC itself. These cells also constitutively express the enzymes required to convert AGT to the processed peptide ANGII and ANGII receptors, as evidenced by the demonstration that synthetic AGT protein applied to AECs in serum-free medium was sufficient to induce ANGII receptor-dependent apoptosis (12).

Fig. 7. Prevention of MG132-induced nuclear fragmentation in alveolar epithelial cells by angiotensin 1–7 or by angiotensin nonselective receptor blockade. A549 cells (A) or primary cultures of human alveolar epithelial cells (B) were treated with MG132 in the presence or absence of saralasin (50 μg/ml), angiotensin 1–7 (10^{-7} M), or the specific mas antagonist A779 (10^{-7} M) for 20 h and were then processed for microscopic quantitation of nuclear fragmentation with propidium iodide (13, see inset in A). Arrow denotes fragmented nucleus amid 3 normal nuclei. Note partial inhibition of nuclear fragmentation by saralasin, complete inhibition by angiotensin 1–7, and reversal of ANG1–7 inhibition by A779. Bars are means ± SE of n = 6 in at least 4 cell cultures; *P < 0.05 vs. CTL, **P < 0.05 vs. MG132 and CTL, ***not significant vs. CTL but P < 0.05 vs. MG132 by ANOVA and Student-Newman-Keuls post hoc test. See MATERIALS AND METHODS for details.

Fig. 8. Abrogation of SP-C mutation-induced nuclear fragmentation in alveolar epithelial cells by angiotensin 1–7 or by angiotensin nonselective receptor blockade. A549 cells were transfected with plasmids containing wild-type (w.t.) human SP-C (21 kDa) or the SP-C BRICHOS domain mutant G100S (17). Immediately after transfection, cells were challenged with saralasin (50 μg/ml), angiotensin 1–7 (10^{-7} M), or the specific mas antagonist A779 (10^{-7} M) for 28 h followed by harvesting for Western blotting of SP-C (A) or processing for nuclear fragmentation assay (B). Note equal levels of SP-C overexpression in both wild-type and G100S-transfected cells (A) and complete blockade of G100S-induced nuclear fragmentation by either saralasin or angiotensin 1–7 but reversal of the ANG1–7 effect by A779 (B). Bars are means ± SE of n = 6 in at least 3 cell cultures; ***P < 0.05 vs. wild-type, *P < 0.05 vs. G100S and ***P < 0.05 vs. G100S+ANG1–7 by ANOVA and Student-Newman-Keuls post hoc test. See MATERIALS AND METHODS for details.
ANGIOTENSIN 1–7 ABROGATES APOPTOSIS INDUCED BY ER STRESS

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Fig. 9. Blockade of the ER stress-induced reduction of ACE-2 and activation of cathepsin D by inhibition of the ACE-2 ectodomain shedding enzyme ADAM17/TACE. A549 cells were treated with ACE-2 (as in Fig. 3), were treated with MG132 as in Fig. 2, or were transfected with plasmids containing wild-type human SP-C or the SP-C BRICHOS domain mutant G100S as in Fig. 8. Thirty minutes before addition of CLBL (10 μM) or MG132 (10 μM) or immediately after transfection, the cells were treated with the ADAM17/TACE-specific inhibitor TAPI-2 (0.8 μM, 4) or vehicle (CTL) and were thereafter harvested for Western blotting. A: inhibition of CLBL-induced ACE-2 loss by TAPI-2. Note TAPI-2-induced increase in high MW form of ACE-2 (arrowhead, 115 kDa) and reversal of the loss of low MW form (88 kDa). B: inhibition of G100S-induced loss of all 3 isoforms of ACE-2 by TAPI-2. Bars are means ± SE of at least 3 cell cultures; **P < 0.05 vs. wild-type and *P < 0.05 vs. G100S by ANOVA and Student-Newman-Keuls post hoc test. C: inhibition of MG132-induced cathepsin D activation by TAPI-2. The low MW (active forms, see Fig. 2) of cathepsin D were quantitated by densitometry relative to β-actin. Bars are means ± SE of at least 3 cell cultures; *P < 0.05 vs. CLBL or MG132. See MATERIALS AND METHODS for details.

ER STRESS
(MG132, mSP-C)

ER STRESS-
induced apoptosis of alveolar epithelial cells. Induction of ER stress activates cathepsin D (CAT-D), one of the enzymes capable of producing ANGII by cleaving angiotensinogen (AGT) synthesized by either AECs or underlying myofibroblasts (10). ER stress also decreases ACE-2, the primary pathway for ANGII generation, even in the absence of an increase in AGT transcription, at least in studies of bleomycin-induced apoptosis of AECs (20). On the other hand, a decrease in ACE-2 (Fig. 3), when achieved artificially through application of a synthetic competitive inhibitor (DX6000) or by siRNA knockdowns, was sufficient to induce apoptosis in AECs without any change in AGT transcription or cathepsin D activity (23). Thus either one of the enzyme alterations induced by ER stress in the present study have the potential to explain the induction of apoptosis by ER stress.

On the other hand, the ability of ANG1–7 to abrogate nuclear fragmentation more potently than saralasin (Fig. 7), as well as some apoptosis signaling markers such as mitochondrial membrane potential and JNK phosphorylation (Figs. 5 and 6), suggests that the ANG1–7/mas pathway is a more potent regulator of ER stress-induced apoptosis than is ANGII production and binding to ANG receptors. The possibility exists that AT1- or AT2-selective ANG receptor blockers might give more potent inhibition of ER stress-induced AEC apoptosis than saralasin. However, this outcome seems unlikely in the light of earlier studies showing AT1-selective antagonist blockade of apoptosis in response to bleomycin (13), but it would nonetheless be an interesting topic for future studies.

To date, little is known about the regulation of the protective enzyme ACE-2. A recent report by our group found that ACE-2 expression by AECs is highly dependent on cell cycle status, with the highest expression being exhibited by quiescent cells but loss of ACE-2 expression occurring with induction of cell proliferation (21). Given the high fraction of AECs that are proliferating in the fibrosing human lung (i.e., the “hyperplastic epithelium” in IPF), it was speculated that the previously observed loss of ACE-2 expression in the lungs of patients with IPF might be due simply to the high fraction of AECs that are proliferating. This mechanism cannot, however, explain the results reported here because two different proteasome inhibitors (MG132 and CLBL, Fig. 3) each downregulated ACE-2 and did so at two different cell densities, each cultured in the absence of growth factors (serum-free medium). Moreover, the proteasome inhibition caused by these agents is known to inhibit, not stimulate, the cell proliferation that downregulated ACE-2 in the absence of these agents (21). For this reason, it is suggested here that ER stress constitutes another mechanism responsible for the downregulation of ACE-2 in fibrotic human lung.

Fig. 10. Roles of the autocrine angiotensin system in ER stress-induced apoptosis of alveolar epithelial cells. Induction of ER stress activates cathepsin D (CAT-D), one of the enzymes capable of producing ANGII by cleaving angiotensinogen (AGT) synthesized by either AECs or underlying myofibroblasts (10). ER stress also decreases ACE-2, the primary pathway for ANGII degradation that normally generates the antiapoptotic heptapeptide ANG1–7. ER stress-induced apoptosis of AECs in response to either proteasome inhibition (MG132) or the SP-C BRICHOS domain mutant G100S (17) can be prevented by either ANG receptor blockade (saralasin) or by administration of ANG1–7. Activation of the ANG1–7 receptor mas reduces ANGII receptor-mediated JNK phosphorylation by unknown mechanisms (?) that are currently under investigation.
The finding that TAPI-2, an inhibitor of ADAM17/TACE (TNF-α converting enzyme, Fig. 9), prevented the CLBL- and G100S-induced loss of cellular ACE-2 suggests that ACE-2 ectodomain shedding constitutes another mechanism by which ACE-2 is reduced in fibrotic human lung (11). Indeed, ER stress from a variety of stimuli was already shown to induce ADAM17/TACE and ectodomain shedding of ACE-2 (18), albeit in non-pulmonary cell types. By analogy, the findings reported in Fig. 9 strongly suggest that the general mechanism of ADAM17-induced ACE-2 ectodomain shedding might occur in lung fibrogenesis in response to various stimuli that could include viral infection, mutant proteins, or even hyperoxic gas (18), each of which would be compelling topics for further inquiry. In addition, the finding that TAPI-2 also reduced the active forms of cathepsin D (Fig. 9C) is, to our knowledge, the first evidence to suggest a role for ADAM17/TACE, or related enzymes that might be inhibited less potently by TAPI-2 (4), in the enzymatic activation of cathepsin D in response to any stimulus. Each of these theories is currently under investigation.

In summary, the results reported here demonstrate that ER stress of alveolar epithelial cells simultaneously activates ANGII-producing pathways and downregulates ANGII-degrading pathways that normally generate the antiapoptotic peptide ANG1–7. ER stress induced by either a synthetic proteasome inhibitor or by the SP-C BRICHOS domain mutant G100S caused AEC apoptosis that could be blocked by either the ANGII receptor blocker saralasin or, more potently, by the synthetic ANG1–7. The product of the enzyme ACE-2. These data demonstrate that ER stress-induced apoptosis of AECs is mediated by the autocrine ANGII/ANG1–7 system and suggest that administration of ACE-2 or its enzymatic product ANG1–7 may hold therapeutic potential for the treatment of ER stress-induced fibrotic lung disease.

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DISCLOSURES

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REFERENCES


L40 ANGIOTENSIN 1–7 ABROGATES APOPTOSIS INDUCED BY ER STRESS


