Accumulation of Mutant $\alpha_1$-Antitrypsin Z in the Endoplasmic Reticulum Activates Caspases-4 and -12, NF$\kappa$B, and BAP31 but Not the Unfolded Protein Response

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In $\alpha_1$-antitrypsin ($\alpha$1AT) deficiency, a polymerogenic mutant form of the secretory glycoprotein $\alpha$1AT, $\alpha$1ATZ, is retained in the endoplasmic reticulum (ER) of liver cells. It is not yet known how this results in liver injury in a subgroup of deficient individuals and how the remainder of deficient individuals escapes liver disease. One possible explanation is that the “susceptible” subgroup is unable to mount the appropriate protective cellular responses. Here we examined the effect of mutant $\alpha$1ATZ on several potential protective signaling pathways by using cell lines with inducible expression of mutant $\alpha$1AT as well as liver from transgenic mice with liver-specific inducible expression of mutant $\alpha$1AT. The results show that ER retention of polymerogenic mutant $\alpha$1ATZ does not result in an unfolded protein response (UPR). The UPR can be induced in the presence of $\alpha$1ATZ by tunicamycin excluding the possibility that the pathway has been disabled. In striking contrast, ER retention of nonpolymerogenic $\alpha$1AT mutants does induce the UPR. These results indicate that the machinery responsible for activation of the UPR can distinguish the physical characteristics of proteins that accumulate in the ER in such a way that it can respond to misfolded but not relatively ordered polymeric structures. Accumulation of mutant $\alpha$1ATZ does activate specific signaling pathways, including caspase-12 in mouse, caspase-4 in human, NF$\kappa$B, and BAP31, a profile that was distinct from that activated by nonpolymerogenic $\alpha$1AT mutants.

In the classical form of $\alpha$1AT$^2$ deficiency, the mutant $\alpha$1ATZ molecule is retained in the endoplasmic reticulum (ER) of liver cells rather than secreted. There is an 85–90% reduction in $\alpha$1ATZ levels in the blood and body fluids. This deficiency affects ~1 in 1800 live births and results in the premature development of pulmonary emphysema in adult life. Chronic liver disease develops in a subgroup of homozygotes, usually becoming evident during childhood. There is also an increased incidence of hepatocellular carcinoma later in life. Emphysema is caused by a loss-of-function mechanism whereby lack of $\alpha$1AT in the lung allows proteolytic destruction of the connective tissue matrix. In contrast, liver injury appears to involve a gain-of-toxic-function mechanism whereby the accumulation of mutant $\alpha$1ATZ in the ER damages liver cells (1).

Nevertheless, relatively little is known about the factors that predispose the “susceptible” subgroup of PIZZ individuals to liver disease and/or protect the remainder of the PIZZ population from liver disease. By using skin fibroblast cell lines from PIZZ individuals with or without liver disease engineered for expression of $\alpha$1ATZ, we have shown previously that there is a lag in ER degradation of mutant $\alpha$1ATZ in cells from PIZZ individuals with liver disease (2). These results provided evidence that the response of cells to the accumulation of this mutant protein in the ER, particularly the degradative machinery, could play a role in determining the susceptibility to liver disease among affected individuals. We now know that degradation of mutant $\alpha$1ATZ is mediated by several pathways, including the proteosome, autophagy, as well as one or more nonproteosomal mechanisms (1).

In the absence of efficient protein degradation or if accumulation of misfolded proteins in the ER overwhelms the degradative machinery, several ER response pathways can be activated. For example, the unfolded protein response (UPR) is a signal transduction pathway activating a wide spectrum of genes in response to the accumulation of unfolded, misfolded, or unassembled proteins in the ER. In addition to new synthesis of ER chaperones and enzymes that facilitate disulfide bond formation, bolstering the protein-folding capacity of the ER, and lipids for synthesis of new ER membrane required to handle the increased protein load, an increase in the synthesis of proteins that constitute the ER degradative machinery, the glycosylation system, and other cellular transport mechanisms occurs. There is also a decrease in initiation of translation in such a way that only specific mRNAs can be translated (3, 4). The prototype target of the UPR is the ER chaperone GRP78/BiP. Transcriptional activation results in marked increases in BiP levels in the ER presumably to facilitate folding as a protective mechanism.

We now know that at least three ER-localized transmembrane proteins, Ire1, ATF-6, and PERK, constitute the proximal transducers of the UPR (5). Binding of BiP to the luminal domain of these proteins is responsible for maintaining them in an inactive monomeric state. During ER stress, BiP is titrated away to bind to misfolded proteins. For Ire1 this results in dimerization and activation of the endoribonuclease properties in its cytosolic tail. Activated Ire1 alters the processing of XBP-1 mRNA in such a way that a larger transcript and protein product with transcriptional activity is generated. Spliced XBP-1 can activate the transcription of a number of UPR target genes. For ATF-6, BiP dissociation allows it to move to the Golgi where it is cleaved. The cytosolic fragment of ATF-6 has direct transcriptional activating properties for UPR target genes, including XBP-1 itself, the transcription factor CHOP, and ER chaperones such as BiP and GRP94. For PERK, disoci-
ation of BiP results in dimerization and activation of a cytosolic kinase that attenuates protein translation by phosphorylation of eIF-2α.

Several other responses to ER retention of misfolded proteins have been described. First, Pahl et al. (6) have described the activation of NFκB in cells treated with tunicamycin, 2-deoxyglucose, or brefeldin A and in cells in which the adenovirus E3/19K protein is retained in the ER. This signaling pathway, which appears to involve ER calcium release, has been called the ER overload pathway because it appears to be distinct from the UPR. Second, Nakagawa et al. (7) have shown that treatment of mouse cells with drugs that cause ER stress, including tunicamycin, thapsigargin, A23187, and brefeldin A, results in the activation of an ER-specific caspase, caspase-12. Third, Hitomi et al. (8) have shown recently that caspase-4 is activated in human cells under ER stress. Fourth, BAP31 is an integral membrane protein of the ER that appears to be involved in the retention of several proteins in the ER (9). Cleavage of BAP31 can direct pro-apoptotic signals from the ER to mitochondria (10). Although there is no evidence that ER stress mediates it, cleavage of BAP31 may be particularly relevant to the pathologic state that develops in α1AT deficiency with accumulation of mutant α1ATZ in the ER because that state has been shown to be associated with mitochondrial dysfunction (11).

In order to understand how cells respond to the retention of mutant α1ATZ in the ER, we recently developed model cell lines and transgenic mice with inducible expression of α1ATZ. In contrast to previous studies using transfected cell lines or transgenic mice with constitutive expression of α1ATZ in which it is possible that only cells that had "adapted" to retention of α1ATZ in the ER survive the selection process, the inducible systems presumably allow us to characterize the very first response of cells to ER retention of α1ATZ before they have the opportunity to "adapt" and then to actually characterize the subsequent adaptations as they occur. Because it is possible to establish inducible expression in stable transfected cell line, this type of system is also superior to transient transfected systems that may be compromised by experiment-to-experiment variation in transfection efficiency and the inherent cell toxicity associated with transfection. Here we used these cell lines and mice to determine which signal transduction pathways are activated by accumulation of mutant α1ATZ in the ER.

EXPERIMENTAL PROCEDURES

Materials—Antibody to human α1AT was purchased from DAKO (Santa Barbara, CA). Antibodies to BiP (SC1050), GRP94 (SC 1794), CHOP/GADD153, IRE1, and PERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to caspase-12 were purchased from Cell Signaling Technology (Cambridge, MA) and were kindly provided by Dr. J. Yuan (Boston, MA). Antibody to BAP31 was kindly provided by Dr. G. Shore (Montreal, Quebec, Canada). Antibody to tTA (TetR monoclonal antibody) was purchased from Clontech. Antibodies to caspase-4 and ATF-6 were from Imgenex (San Diego, CA). Human liver originated from excess liver tissue of a normal liver transplant donor and from the native liver of two patients with homozygous α1AT deficiency who underwent liver transplantation.
Cell Lines—In order to establish cell lines with tightly regulated expression of α1AT, we used the human epidermal cell line HeLa, because it does not express the endogenous α1AT gene, and the Tet-Off inducible system, because it provides low background expression and high inducibility of expression of target genes. HeLa Tet-Off cells (HTO) were obtained from Clontech. Wild type α1AT, mutant α1ATZ, mutant α1AT Saar, and mutant α1AT SaarZ cDNA inserts (12) were subcloned downstream of the tetracycline-response element in the pTRE plasmid from Clontech. These plasmids were then used to transfect the HTO cells described above. Subclones from these transfections were tested for expression of α1AT by radioimmunoprecipitation of cell lysates and analysis by SDS-PAGE/fluorography. For induction of α1ATZ in cells of hepatocytic origin, we used the murine hepatoma Hepa1-6. Hepa1-6 was first engineered for expression of tetracycline-controlled transactivator tTa using the pTet-Off plasmid from Clontech. Luminescent assays after transient transfection of the pTRELuc plasmid from Clontech were used to select a subclone with the lowest background and highest inducibility for expression of the reporter. This subclone was called HepaTO. The pTRE α1ATZ, pTRE α1AT Saar, pTRE α1AT SaarZ, and pTRE α1AT wild type plasmids were used to transfect the HepaTO subclone. Subclones from the second round of transfection were tested in the same way as the HeLa cell lines described above. CHO-K1 cell lines that express wild type α1AT and the three different α1AT mutants have been described previously (12).

Transgenic Mice—To examine the effects of ER α1ATZ in vivo, we generated transgenic mice with liver-specific inducible expression of α1AT. We used the approach of Bujard’s lab with tetracycline suppressing expression of a target gene (13). This system is designed for low background levels of expression and high levels of inducibility of expression of the target gene in liver. First, we generated “target” transgenic mice with wild type α1AT, mutant α1ATZ, mutant α1AT Saar, and mutant α1AT SaarZ cDNA downstream of the Tet-response element in plasmid pTRE as transgene. FVB/N mice were used because they are an inbred line well characterized and superior in fertility. Fertility were screened by PCR analysis of tail DNA. Founders that were positive for the transgene were mated to murine mammary tumor virus-CAMK2tTa mice from The Jackson Laboratory. The murine mammary tumor virus-CAMK2tTa mice are transgenic for a plasmid that encodes the tTA tetracycline transactivator under the control of the P<sub>lap</sub> promoter that directs liver-specific transcription (13). Mice with inducible liver-specific expression of the wild type α1AT gene were called M mice, and those with inducible liver-specific expression of mutant α1ATZ were called Z mice. We also generated Saar and SaarZ mice in the same way. PiZ mice (14) were bred into the C57BL6 background (15, 16).

Radioimmunoprecipitation, Sucrose Density Gradient Centrifugation, and Western Blot Analysis—Bio synthetic labeling, immunoprecipitation, and SDS-PAGE/fluorography followed previously published protocols (17). For sucrose density gradient centrifugation, cells grown to confluence were homogenized under nondenaturing conditions in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5% Triton X-100, 0.5% sodium deoxycholate, 2 mM N-ethylmaleimide supplemented with complete protease inhibitor mixture (Roche Applied Science) and 2 mM phenylmethylsulfonyl fluoride as described previously (18, 19). After homogenization, cell homogenates were cleared of debris by a 5-min centrifugation at 3000 × g, and then the cleared lysate was loaded on top of a 5–60% sucrose gradient prepared in 14 × 89-mm tubes. Gradients were centrifuged in a Beckman SW 41 rotor at 28,500 rpm for 18 h at 4 °C, and then 500-µl fractions were collected from the top. The refractive index of fractions was measured in order to calculate their sucrose concentrations. Fractions were split into two, and 1 aliquot was immunoprecipitated, whereas proteins were precipitated with trichloroacetic acid from the other. Immunoprecipitates or resolubilized trichloroacetic acid precipitates were separated on 10% SDS-PAGE and transferred to supported nitrocellulose for Western blot analysis.

For Western blot analysis to detect α1AT or BiP in the HeLa cell lines, cells were lysed in phosphate-buffered saline, 1% Triton X-100, 0.5% deoxycholic acid, and 2 mM phenylmethylsulfonyl fluoride. To detect α1AT or BiP in the Hepa1-6 cell lines, cells were lysed in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5% deoxycholic acid, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride supplemented with complete protease inhibitor mixture (Roche Applied Science). To detect caspase-12, caspase-4, or BAP31, cells were lysed in 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 0.72 mM β-mercaptoethanol, and 7% glycerol. To detect cleavage of ATF-6 and phosphorylation of IRE1, we...
used the conditions of Lee et al. (20) for Western blot analysis. To detect phosphorylation of PERK, we followed the Western blot protocol of Xue et al. (21). In each case cells treated with tunicamycin were used as a positive control. Equivalent amounts of protein as determined by the BCA assay (Pierce) were loaded onto SDS-polyacrylamide gels. Blots were incubated with primary antibody and then with peroxidase-labeled secondary antibody and were developed with the Supersignal chemiluminescent substrate (Pierce).

**Gel Shift Assays**—Nuclear extracts were prepared using high salt buffer in the chemical nuclear and cytoplasmic extraction reagents kit (Pierce) and then incubated with 32P-labeled oligonucleotides that contained the NFκB consensus sequence using the gel shift assay system kit from Promega. Oligonucleotides were labeled with γ-32P]ATP by T4 polynucleotide kinase. Native 4% (w/v) polyacrylamide gels were used to analyze the reactions.

**XBP1 mRNA Processing**—XBP1 mRNA processing was assessed by an RT-PCR assay (22). RNA was harvested from the liver of Z and M mice drinking water that was supplemented with dox or water from which dox had been withdrawn 3 weeks prior to the assay. RNA was also harvested from M mice that were treated with tunicamycin by using the protocol of Zinszner et al. (23). Brieﬂy, tunicamycin in a 0.2 mg/ml 150 mM dextrose solution was administered intraperitoneally in several different doses to 7-week-old M mice. Control mice were injected intraperitoneally with 150 mM dextrose. The mice were sacrificed 8 h later and livers harvested for RNA.

**RESULTS**

**A Cell Line with Inducible Expression of α1ATZ**—In order to determine whether there is inducible expression of α1ATZ in the HeLa cell line HTO/Z, engineered for expression of the target gene when the tetracycline analogue dox is removed from the cell culture fluid, we first examined synthesis of α1ATZ in this cell line. HTO/Z cells that had been maintained in the absence of dox were incubated for 3 days in medium supplemented with several doses of dox and then were pulse-labeled (Fig. 1a). Synthesis of the ~52-kDa α1AT polypeptide was suppressed by dox in a concentration-dependent manner with complete abrogation at a concentration of 1 ng/ml.

Next, HTO/Z cells were incubated in medium supplemented with 1.0 ng/ml dox for several different time intervals (Fig. 1b, upper panel). Synthesis of α1ATZ decreased in a time-dependent manner with almost complete disappearance by 48 h. There was no suppression of the synthesis of the Tet transactivator (Fig. 1b, lower panel), indicating that the regulatory effect on α1ATZ was highly specific.

Next, HTO/Z cells that had been maintained in dox were incubated with dox-free medium for several different time intervals (Fig. 1c). The results show induction of α1ATZ within 2 days, increasing progressively from 7 to 17 days and then plateauing between 17 and 27 days. Taken together, these results indicate that expression of α1ATZ in the HTO/Z cell line is concentration-dependent, time-dependent, and reversible.

In order to determine whether the HTO/Z cell line faithfully recapitulated the defect that characterizes α1AT deficiency, it was compared with the HTO/M cell line in a pulse-chase experiment (Fig. 1d). In HTO/M cells (Fig. 1d, top panel), wild type α1AT is synthesized as a 52-kDa polypeptide. It is converted to a 56-kDa polypeptide by 15 min of the chase period coincident with the appearance of the 56-kDa polypeptide in the medium. Both the 52- and 56-kDa polypeptides disappear intracellularly (IC) between 30 and 60 min of the chase as the mature 56-kDa polypeptide increases EC. In HTO/Z cells, the 52-kDa polypeptide has retained IC with very little disappearance even by 120 min of the chase period and a much lesser amount of the 56-kDa polypeptide appearing at later time points EC. Immunofluorescence studies indicated that α1ATZ co-localized with the ER protein calnexin (data not shown). These results indicated that the HTO/Z cell line is an appropriate model for α1AT deficiency.

**Effect of Inducing α1ATZ Expression on the UPR**—In order to determine whether the UPR is activated when the α1ATZ gene is induced in HTO/Z cells, these cells were examined 7 days after dox was removed from the medium (Fig. 2a). The results show that the 52-kDa α1ATZ polypeptide only appears when dox is removed from the medium. However, there is no change in synthesis of BiP. An ~78-kDa doublet is present in equivalent amounts when dox is in the medium or withdrawn from the medium. One interesting observation from these studies is the presence of a 52-kDa polypeptide that co-precipitated with BiP when
dox is withdrawn from the medium. This polypeptide co-migrates with α1ATZ, and the result implies that α1ATZ binds to BiP after its expression is induced in HTO/Z cells. The ~78-kDa doublet was seen in all of our cell lines when BiP was immunoprecipitated from pulse-labeled cells. Immunoprecipitation of both bands by anti-BiP was blocked by unlabeled purified BiP (see supplemental Fig. 1). The top band of the doublet is increased when the UPR is activated (see Fig. 7, c and d, below) and is the band that is co-precipitated with mutant α1AT by anti-α1AT (see supplemental Fig. 2). The lower band is not a precursor or a product of the upper band as determined by pulse-chase experiments (data not shown). Further studies, including sequence analysis, will be necessary to determine the exact molecular nature of the lower band of this doublet. The HTO/Z cells are capable of inducing the UPR as shown in Fig. 26 by the time-dependent induction of BiP synthesis and the increase in GRP94 synthesis when the cells are subjected to glucose starvation, a known stimulus for the UPR.

HTO/Z cells were also examined at earlier time points for up-regulation of BiP synthesis. In Fig. 2c, synthesis of α1ATZ is apparent within 3 days of withdrawal of dox, but there is no increase in synthesis of BiP before or after that time. Synthesis of BiP did not increase at later times either. In Fig. 2d there is no change in synthesis of BiP at 10 or 17 days after withdrawal of dox. RT-PCR assays showed no change in BiP or GRP94 mRNA levels in HTO/Z cells 4, 7, or 14 days after withdrawal of dox (data not shown).

In order to examine the possibility that higher levels of expression of α1ATZ would lead to activation of the UPR, we generated several additional clones of HeLa Tet-Off cells with inducible expression of α1ATZ. This time we used a higher dose of dox during transfection and selection presumably to more completely suppress expression of α1ATZ. The clone with the highest level of expression was called HTO/Z2. In Fig. 3a, HTO/Z2 cells were analyzed for α1AT. The results show that synthesis of α1ATZ is well over 10-fold higher in HTO/Z2 than HTO/Z. Immunoprecipitation for BiP shows no change in levels of the 78-kDa doublet. Thus, there is no activation of BiP synthesis. Co-precipitation with BiP of the 52-kDa polypeptide that corresponds to α1ATZ is also more apparent in HTO/Z2 than HTO/Z cells and is only seen when α1ATZ expression is induced by withdrawal of dox. Western blot analysis did not detect phosphorylation of IRE1 or PERK or cleavage of ATF-6 when dox was removed from, and α1ATZ expressed in, the HTO/Z2 cell line (data not shown).

In order to examine the possibility that expression of α1ATZ in a hepatocytic cell line would lead to activation of the UPR, we generated a mouse hepatoma Hepa1-6 cell line with inducible expression of α1ATZ. In Fig. 3b, we subjected this cell line to Western blot analysis for human α1AT and murine BiP. The results show that expression of α1ATZ is only apparent when dox was withdrawn but again there is no change in steady state levels of BiP.

Expression of BiP in the Liver of Transgenic Mouse Models of α1AT Deficiency and Human with α1AT Deficiency—To examine the possibility that the UPR is induced in the liver in vivo, we determined steady state levels of BiP in two transgenic models of α1AT deficiency. One model is the PiZ mouse that has constitutive expression of α1ATZ in the liver and many other tissues (14, 16). The PiZ mouse has been bred into the C57BL6 background and, therefore, was compared with C57BL6. The second model is the Z mouse with inducible liver-specific expression of α1ATZ. It was made in the FVB/N background. By using this model, we could exclude the possibility that the UPR had been attenuated in liver after a putative adaptive response. In Fig. 4a, Western blot analysis of liver from these mice shows that human α1AT expression in the Z and M mouse is suppressed by doxycycline in the drinking water to undetectable levels over 2 weeks in a time-dependent manner. The α1AT polypeptide detected in the Z and M mouse has an identical electrophoretic mobility to that in the PiZ mouse. α1AT was not detected in the FVB/N mouse providing confirmation that the assay does not recognize the endogenous murine α1AT orthologue. Western blot analysis for murine BiP in Fig. 4b shows that there is essentially no difference in steady state levels of BiP in the liver of two different PiZ mice as compared with C57BL6 and no induction of BiP in either the M or the Z mouse liver after withdrawal of dox from the drinking water. Next, we examined steady state levels of BiP in livers from patients with homozygous PIZZ α1AT deficiency (Fig. 4c). Here again, there was no evidence for increased steady state levels of BiP.

Effect of Tunicamycin on the UPR in Cells Expressing α1ATZ—Here we examined the possibility that ER retention of α1ATZ in the inducible cell lines has a disabling effect on the UPR. For this, the HTO/M and HTO/Z cell lines were treated with tunicamycin, a known activator of the UPR (Fig. 5a). The results show that α1AT is induced when dox is removed from the medium in each case. The level of α1AT synthesis is not affected even though wild type α1AT and mutant α1ATZ migrate faster in the presence of tunicamycin, in each case to a degree completely explained by the lack of oligosaccharide side chain addition (Fig. 5, left panels). Withdrawal of dox has no effect on synthesis of BiP, but tunicamycin mediates an increase in each of the cell lines (Fig. 5, center panels). The increase in synthesis of BiP induced by tunicamycin is predominantly in the top band of the doublet in an identical manner in the two cell lines. The increase in BiP synthesis is identical in the presence or absence of dox, indicating that ER accumulation of α1ATZ does not have a disabling effect on the UPR and that it does not have additive/synergistic effects on induction of the UPR. Results were identical when HepaTO/Z cells were studied in the presence or absence of dox for time.
**FIGURE 5.** a, effect of tunicamycin on synthesis of BiP and CHOP in the absence or presence of mutant α1ATZ gene expression. HTO/M and HTO/Z2 cells were incubated for 7 days in the presence or absence of dox and then treated for 3 h with 2.5 μg/ml tunicamycin. The cells were then pulse-labeled for 60 min, and the resulting cell lysates were analyzed for α1AT (left), BiP (center), and CHOP (right). b, effect of calcium ionophore A23187 on the synthesis of BiP. HTO/Z2 cells were incubated for 3 h in medium supplemented with A23187 in doses indicated at the bottom. The cells were then pulse-labeled for 60 min and analyzed for BiP synthesis. c, effect of tunicamycin and A23187 on secretion of α1ATZ. HTO/Z2 cells that had been out of dox for 7 days were preincubated for 3 h in control (top), 2.5 μg/ml tunicamycin (middle), or 0.25 μM A23187 (bottom) and then subjected to a pulse-chase protocol. The pulse was 60 min.
ER Retention of Mutant α1ATZ Does Not Induce the UPR

One possible problem with these experiments is that by activating the UPR, tunicamycin mediates an increase in the degradation of α1ATZ and, in so doing, eliminates or reduces the ER retention state. In order to address this potential issue, we examined the effect of tunicamycin and another activator of the UPR, calcium ionophore A23187, on the fate of α1ATZ in pulse-chase experiments. First we determined the concentration of A23187 that was necessary for activation of the UPR in HTO/Z cells (Fig. 5b). The results show that synthesis of BiP is increased at doses of 0.125 μM A23187. Next we examined the effect of tunicamycin and A23187 on the fate of mutant α1ATZ in HTO/Z cells (Fig. 5c).

In control HTO/Z cells, the 52-kDa α1ATZ polypeptide accumulates IC over 1 h and then progressively disappears over 3–5 h with only a trace amount of the 55-kDa mature polypeptide appearing extracellularly by 3 h of the chase period. In the presence of tunicamycin, the 46-kDa unglycosylated α1ATZ polypeptide is also retained through 1 h of the chase period and disappears even more slowly over 3–5 h of the chase period, and none of the α1ATZ is secreted. In the presence of A23187, there is even slower disappearance of the 52-kDa α1ATZ polypeptide from IC. A similar amount of mature α1ATZ polypeptide appears in the EC in the presence of A23187 as compared with control. Together, these data indicate that tunicamycin and A23187 do not mediate an increase in degradation of α1ATZ or reduce the ER α1ATZ retention state.

Effect of Accumulation of α1ATZ in the ER on XBP1 mRNA Processing—In order to examine the possibility that BiP synthesis is a relatively insensitive measure of UPR activation, we examined the effect of ER α1ATZ accumulation on XBP1 mRNA processing. Altered processing of XBP1 mRNA is one of the first steps in the signaling pathway that constitutes the UPR, occurring right after activation of IRE1. We used liver from the M and Z mouse and a PCR-based assay (22) for detecting different XBP1 transcripts. First, we examined the liver of M mice that were given tunicamycin as a positive control (Fig. 6a). Altered processing of XBP1 mRNA was detected at three different doses of tunicamycin. Next we examined the effect of withdrawing dox from the drinking water of the Z mouse (Fig. 6b). There was no alteration of XBP1 mRNA processing after withdrawal of dox. There was also no evidence of altered processing of XBP1 mRNA in the PiZ mouse with constitutive expression of α1ATZ. Finally, microarray studies on the HTO/Z cells and on liver from the Z mouse do not show changes in expression of any of the known target genes of the UPR.3 The results of these studies therefore provide even more powerful evidence that the UPR is not activated by ER α1ATZ accumulation.

Effect of Inducing Expression of Other α1AT Mutants on the UPR—Next, we examined the possibility that the UPR is not activated by ER retention of mutant α1ATZ because α1ATZ polymerizes and aggregates. In order to address this, we established HeLa cells with inducible expression of α1AT Saar and α1AT SaarZ, naturally occurring α1AT mutants that are retained in the ER but do not polymerize (12, 19). α1AT Saar is a truncation mutant, missing 19 amino acids at the carboxyl terminus of α1AT, and α1AT SaarZ is a double mutant with the amino acid substitution that characterizes α1ATZ and the truncation that characterizes α1AT Saar. In Fig. 7a, the HTO/Saar and HTO/SaarZ cell lines that we generated were pulse-labeled. The results show that expression of α1AT Saar and α1AT SaarZ is only present when dox is withdrawn. The α1AT Saar and α1AT SaarZ polypeptides migrate slightly faster than wild type α1AT and mutant α1ATZ, because of the truncation.

In previous studies using mechanical shearing and centrifugation, we have provided evidence that the α1AT Saar and α1AT SaarZ do not polymerize, we subjected cell lysates from the HTO cell lines to sucrose density gradient centrifugation under non-denaturing conditions. Fig. 7b shows a Western blot of fractions from sucrose density gradients prepared from lysates of HTO/Z and HTO/SaarZ cells. The results for α1AT Saar show a peak from 8 to 32% sucrose and then another peak at 56% sucrose and in the pellet. Detailed studies have shown that the peak at 8–32% sucrose corresponds to α1ATZ in heterogeneous soluble complexes with multiple chaperones, including BiP and GRP94, and that the peaks at 56% and in the pellet represent very high molecular weight-insoluble α1ATZ free of any chaperones (19). For α1AT SaarZ there is only a peak at 7–18% sucrose and no evidence of insoluble polymers/aggregates in the fractions corresponding to higher molecular weight. Detailed studies of the peak at 7–18% corresponds to soluble complexes of the mutant α1AT SaarZ in complex with BiP and GRP94 in a manner almost identical to what happens to α1ATZ (19). Results from HTO/M and HTO/Saar cells, for wild type α1AT and mutant α1AT Saar, respectively, were almost identical to these results in HTO/SaarZ cells (data not shown), indicating that α1AT Saar and α1AT SaarZ retained in the ER do not form polymers and providing confirmation that these are indeed nonpolymerogenic mutants. These results have two other important implications. First, they provide further evidence that the HTO/Z cell line is a good model for classic α1AT deficiency in that α1ATZ forms insoluble polymers in it. Second, these results indicate that except for the chaperone-free very high molecular weight insoluble polymers of α1ATZ, this mutant has a

3 T. Hidvegi and D. H. Perlmutter, unpublished observations.
similar fate and interaction with chaperones to that of the nonpolymerogenic α1AT mutants.

Next, we used these cell lines to examine the possibility that ER retention of the nonpolymerogenic α1AT mutants induces the UPR (Fig. 7c). The results show that there is an increase in synthesis of the 78-kDa doublet that represents BiP only in the cells that accumulate α1AT Saar. The increase in BiP synthesis is most apparent in the top band of the doublet. The effect of α1AT Saar on synthesis of BiP here is almost identical to that of tunicamycin in HTO/Saar cells in the presence of dox (Fig. 7c, right panel). The increase in synthesis of BiP was dependent on the amount of α1AT Saar that accumulates in the ER when HTO/Saar cells are incubated in different doses of dox for 14 days (Fig. 7d). Synthesis of BiP was induced in an identical manner upon withdrawal of dox from HTO/SaarZ cells (data not shown). These data show that the UPR is not activated by α1ATZ but is activated by nonpolymerogenic α1AT mutants that are also retained in the ER.

We again observed that a 52-kDa polypeptide co-precipitated with BiP in the HTO/Z cells only when dox had been withdrawn from the medium, implying that α1ATZ had bound to BiP. Most interestingly, a slightly faster migrating ~49-kDa polypeptide co-precipitates with BiP in the HTO/Saar cells only when dox has been withdrawn and expression induced. This polypeptide is identical in electrophoretic migration to the newly synthesized α1AT Saar molecule (see Fig. 7a). Immunoprecipitation of lysates from pulse-labeled HTO/Z cells and HTO/Saar cells with anti-α1AT is shown next to the immunoprecipitation with anti-BiP in supplemental Fig. 2. This shows that the top band of the BiP doublet is co-precipitated with α1AT when anti-α1AT is used in each case and that a similar amount of this top band is co-precipitated with α1ATZ and α1AT Saar. These results militate against the possibility that the differences in activation of the UPR by α1AT Saar as compared with α1ATZ are due to binding of BiP in one case but not the other or due to binding of more BiP in one case but not the other.

**Effect of ER Retention of Mutant α1ATZ on Murine Caspase-12 and Human Caspase-4 Activation**—Treatment of cells with drugs that cause “ER stress,” including tunicamycin, thapsigargin, A23187, and brefeldin A, has been shown to result in activation of an ER-specific caspase, caspase-12, in mouse cells (7). We examined the possibility that ER retention of α1ATZ results in activation of caspase-12 by using our mouse hepatoma cell lines with inducible expression of α1ATZ as well as in the livers of Z mice. Western blot analysis was used to detect the ~35-kDa cleavage product that corresponds to activated caspase-12. The results show that caspase-12 is indeed activated in HepaTO/Z cells but not in HepaTO/M cells (Fig. 8a). Most interestingly, it took a relatively long time for caspase-12 cleavage to become detectable. Cleavage was apparent 22 days after withdrawal of dox but could also be seen in trace amounts by 13 days after withdrawal of dox. This cleavage fragment was seen in the liver of Z mice 2 weeks after dox was removed but was also present in trace amounts in the liver of Z mice on dox (Fig. 8b), suggesting the possibility that dox does not completely suppress α1ATZ expression in the liver of the Z mouse. Indeed, we occasionally observe trace amounts of α1AT synthesis in the presence of dox, but there is still always marked induction of its synthesis in the absence of dox in those cell lines.

Quantitative results are shown in the bottom panel with sucrose concentration of each fraction in the inset. c, synthesis of BiP in cell lines with inducible expression of truncated α1AT mutants. HTO/Vec, HTO/M, HTO/Saar, and HTO/Z cell lines were incubated for 5 days in the presence (+) or absence (−) of dox (left panel). For the right panel, HTO/Saar cells were pretreated for 3 h in the absence or presence of 2.5 μg/ml tunicamycin. The cells were then pulse-labeled for 60 min and analyzed for BiP. d, synthesis of BiP in the HTO/Saar cell line in several different concentrations of dox. The cell line was incubated for 7 days in different concentrations of dox as shown at the bottom of each panel. The cells were then pulse-labeled for 60 min and analyzed for α1AT (left) and BiP (right).
cases (data not shown). Caspase-12 cleavage was not detected in the M mouse on or off dox (Fig. 8b). The same samples were analyzed for steady state levels of BiP (Fig. 8c), confirming previous results that BiP levels are not increased and further verifying the specificity of the caspase-12 activation. We also found that activation of caspase-12 in response to ER accumulation of α1ATZ was dependent on the amount of α1ATZ accumulation. HepaTO/Z cells were incubated for 28 days in different doses of dox and were then subjected to Western blot analysis for caspase-12. The results show that activation of caspase-12 is indeed dose-dependent beginning with the lowering of dox to 0.4 and 0.1 ng/ml and then no dox (data not shown).

Recent studies have suggested that caspase-4 is the ER stress-specific caspase in humans (8), so we examined the possibility that accumulation of α1ATZ in the ER of human cells activates caspase-4. HTO/M and HTO/Z2 cells were subjected to Western blot analysis for caspase-4 (Fig. 8d). The results demonstrate ~35-kDa pro-caspase-4 in HTO/M and HTO/Z2 cells but cleavage to an ~35-kDa fragment only in HTO/Z2 cells. The cleavage was evident in trace amounts in HTO/Z2 cells that had been maintained in dox, suggesting the possibility that there is a low level of expression of α1ATZ (below the limits of detection) in the suppressed state. However, there is an increase in the cleavage fragment within 5 days after withdrawal of dox and further increases from 5 to 36 days later.

Effect of ER Retention of Mutant α1ATZ on NFκB Activation—Next, we examined the possibility that ER retention of α1ATZ activated the ER overload pathway by using our mouse hepatoma cell lines and classical gel shift assays for NFκB DNA binding activity (Fig. 9a). The results show marked induction of DNA binding activity for NFκB in HepaTO/Z cells by 28 days after withdrawal of dox. The activity was comparable with that of the positive control A23187. It was not detected in HepaTO/M cells (Fig. 9b). The DNA binding activity in HepaTO/Z was supershifted by antibody to p50, blocked by unlabeled NFκB oligonucleotides but not by unlabeled AP-2 oligonucleotides (Fig. 9b).

Next, we examined the possibility that NFκB was activated in the liver of the Z and PiZ mouse models of α1AT deficiency. The results show that there is an increase in NFκB DNA binding activity in the liver of the Z mouse at 6 weeks of age when dox is not present in the drinking water as compared with the liver of the Z mouse at 6 weeks of age when dox is present and the liver of the M mouse in the presence or absence of dox (Fig. 9c). NFκB activation increased further in the Z mouse liver between 6 and 16 weeks of age in the absence of dox. This time-dependent increase corresponds to increased accumulation of α1ATZ in the liver (data not shown). There is also increased NFκB activity in the liver of the PiZ mouse as compared with the control C57BL6 mouse (Fig. 9c).

In Fig. 9d, the gel shift bands present in the liver of the Z mouse after withdrawal of dox and in the liver of the PiZ mouse are shown to be...
ER Retention of Mutant α1ATZ Does Not Induce the UPR

Because ER retention of α1ATZ does not activate the UPR, these results provide strong evidence for the assertion that the “ER overload pathway” is distinct from the UPR.

Effect of ER Retention of Mutant α1ATZ on Cleavage of BAP31—BAP31 is an integral membrane protein of the ER that appears to be involved in the ER retention of several proteins (9). Cleavage of BAP31 can direct pro-apoptotic signals between the ER and mitochondria (10). Because we have found that ER retention of α1ATZ is associated with mitochondrial dysfunction (11), here we examined the possibility that it mediates the cleavage of BAP31. For this we used our hepatoma cell lines and Western blot analysis for cleavage products of BAP31 (Fig. 10a). The results show an equivalent amount of the ~30-kDa BAP31 precursor in HepaTO/M and HepaTO/Z cells, but the ~20-kDa cleavage product was only detected in HepaTO/Z cells. The effect was of lesser magnitude than that of the positive control, activation of the Fas pathway by anti-Fas antibody in the presence of cycloheximide, and took a relatively long time to evolve. It was clearly present by 40 days but could also be detected in trace amounts by 28 days after withdrawal of dox. Cleavage/activation of BAP31 was also dependent on the amount of α1ATZ that accumulates in the ER. This is shown in Fig. 10b when HepaTO/Z cells were incubated for 28 days in several different doses of dox. The ~20-kDa cleavage product is first seen as the dose of dox is lowered to 0.1 ng/ml and increases further when dox is completely absent. Taken together the results show that activation of BAP31 is a specific effect of the ER α1ATZ retention state.

Effect of ER Retention of Nonpolymerogenic Mutants on Activation of ER Caspase, NFκB, and BAP31—In order to determine whether the nonpolymerogenic mutants also differ in the effect on ER caspases, BAP31, and NFκB, we generated HepaTO cell lines with inducible expression of α1AT Saar and α1AT SaarZ. In Fig. 11a, the HepaTO/Saar cell line was incubated in the presence or absence of dox for 10 days. The results show that synthesis of α1AT Saar is induced and moreover that this is associated with an increase in the synthesis of BiP, consistent with activation of the UPR. In some experiments, a trace amount of α1AT synthesis could be detected in the HepaTO/Saar cell line in the presence of dox but always with a considerable increase in synthesis when compared with that present in the absence of dox (data not shown).

Next we used this cell line to determine whether accumulation of α1AT Saar leads to activation of caspase-12 (Fig. 11b). The results show no evidence for the activation/cleavage of caspase-12 in the HepaTO/Saar cell line in the presence or absence of dox under the same condi-

DNA-binding protein is indicated by an arrowhead at the right margin. b, nuclear extracts from HepaTO/Z cells that had been maintained in dox (−) or off dox for 28 days (−) were subjected to gel shift analysis with labeled oligonucleotides in the absence or presence of anti-p50, unlabeled NFκB oligonucleotides, or unlabeled AP-2 oligonucleotides. A HeLa cell extract was used as positive control. c, nuclear extracts from the liver of Z mice on dox (−) or off dox (−) since birth, C57BL6 (C57) mice, or Piz mice at the age indicated at the top were subjected to gel shift analysis with labeled oligonucleotides. A HeLa extract was used as a positive control. d, nuclear extracts from the liver of Z mice on dox (−) or off dox (−) since birth, C57BL6 (C57) mice, or Piz mice at the age indicated at the bottom of the gel were subjected to gel shift analysis with labeled oligonucleotides in the absence or presence of anti-p50 (SS Ab), unlabeled NFκB oligonucleotides, or unlabeled AP-2 oligonucleotides. The migration of the gel shift band is indicated by the large arrowhead and of the supershift band by the small arrowhead.
ER Retention of Mutant α1ATZ Does Not Induce the UPR

FIGURE 10. Effect of ER retention of α1ATZ on BAP31 cleavage. α, HepaTO/M and HepaTO/Z cells that had been maintained in dox (+) or out of dox for several different time intervals, as indicated at the bottom, with (+) or without (−) treatment with anti-Fas in the presence of cycloheximide exactly as described under "Experimental Procedures," were harvested for Western blot analysis for BAP31. The migration of molecular mass markers is shown at the right margin, and the migration of the ~20-kDa cleavage product is indicated by the arrowhead. β, HepaTO/Z cells were incubated for 28 days in several different doses of dox, including 4, 0.4, and 0.1 ng/ml and none. The cells were then harvested and lysates subjected to Western blot analysis for BAP31.

DISCUSSION

Studies of Pelizaeus-Merzbacher disease (24) and diabetes in the Akita mouse model (25) have provided evidence that cellular responses to mutant proteins that accumulate in the ER can modulate the severity of those diseases. A detailed understanding of the cellular response pathways that are activated by accumulation of mutant α1ATZ in the ER is also likely to be informative because liver injury and hepatocarcinogenesis in α1AT deficiency appear to involve a gain-of-toxic-function mechanism, and because dramatic differences in the liver disease phenotype among individuals affected by this disease raise the possibility that specific cellular response pathways or cellular adaptations account for "protection" or "susceptibility" to liver disease. Previous studies have shown that the autophagic response is activated (15), and in some cases this is accompanied by mitochondrial injury as well (11). In other previous studies, activation of the UPR was not found in the PiZ mouse by examining steady state levels of hepatic BiP (26) or in transiently transfected Chinese hamster ovary cells by examining steady state levels of BiP and transcriptional activation of the BiP promoter (27). However, because each of these systems is engineered for constitutive expression of α1ATZ, it is possible that these results could be affected by compensatory adaptations that permit experimental cell systems or transgenic mice to tolerate the accumulation of a mutant protein in the ER. In this current study we used cell lines and transgenic mice with inducible expression of mutant α1AT in order to ensure that the experimental studies would reflect the response of cells that had not had any opportunity to undergo adaptation and to determine ultimately how cells will respond to this mutant protein at specifically designated concentrations, time intervals, and in the transgenic mice in vivo at specified developmental stages. Although it is true that the mutant protein is expressed constitutively in the human disease in vivo, we know that its expression is regulated by many physiologic and pathophysiologic factors. Furthermore, because ER retention of mutant α1ATZ can be activated at specific ages, durations, and concentrations in the inducible systems, they offer the opportunity to model experimentally the sequence of events that leads to the evolution of the human liver disease. By using these inducible systems, it was possible to provide conclusive evidence that accumulation of mutant α1ATZ in the ER does not activate the UPR. This was shown by examining synthesis of BiP and another target of the UPR, CHOP, in a HeLa cell line with inducible expression of α1ATZ. In order to exclude the possibility that the UPR would be activated by higher levels of expression of mutant α1ATZ, we generated a HeLa cell line with very high levels of inducible expression, and again we demonstrated that ER accumulation of this protein does not activate the UPR. Next we examined the possibility that the UPR would be activated in cells of hepatocytic lineage. There was no evidence for increased steady state levels of BiP in a murine hepatoma cell line engineered for inducible expression of α1ATZ. Steady state levels of BiP were also not increased when α1AT gene expression was induced in the liver of a new mouse model with liver-specific inducible expression of α1ATZ. In order to exclude the possibility that synthesis or steady state levels of BiP were an insensitive measure of the UPR, we investigated the effect of ER retention of α1ATZ on processing of XBP1 mRNA. Alteration in the processing of XBP1 mRNA follows immediately after activation of IRE1 at the ER membrane during the UPR. There was no evidence for processing of XBP1 mRNA in the liver of the Z mouse when α1ATZ was induced or in the liver of the PiZ mouse. We also examined the possibility that accumulation of α1ATZ in the ER has a disabling effect on the UPR. There was no difference in BiP synthesis induced by tunicamycin in HTO/Z2 cells in the absence or presence of α1ATZ expression. Together with the lack of processing of XBP1 mRNA, these results make it unlikely that accumulation of α1ATZ is "sensed" by the machinery of the UPR, but the signal is not transmitted because of a block in the afferent or efferent components of the response pathway. One other possibility that increased levels of BiP were not
detected because a significant portion of the cellular pool of BiP was not measured because it was in insoluble aggregates with α1ATZ was excluded by several observations as follows: lack of increase in CHOP synthesis; lack of increase in BiP mRNA levels; lack of gene expression profile indicative of UPR activation; lack of processing of XBP1 mRNA; and lack of co-precipitation of BiP with insoluble α1ATZ in sucrose density gradient studies under nondenaturing conditions (19).

In order to examine the possibility that it is the polymerogenic properties of α1ATZ that account for the lack of activation of the UPR, we examined the effect of two naturally occurring nonpolymerogenic mutants of α1AT that are retained in the ER. In our previous studies, we showed that the truncated α1AT Saar mutant and the α1AT SaarZ mutant, which bears the mutations that characterize α1ATZ as well as α1AT Saar, were retained in the ER for as long or longer than α1ATZ (12). We could not detect polymers of these two mutants in transfected cells by using a relatively simple shearing and centrifugation assay (12). Here, and in another recent publication (19), we could not detect polymers of these mutants by sucrose density gradient centrifugation in the newly developed cell lines as well as in previously developed cell lines. However, induction of the expression of each of these mutants was associated with activation of the UPR. These data indicate that the lack of activation of the UPR by ER retention of α1ATZ has something to do with the propensity of α1ATZ to form insoluble polymers in the ER.

Lack of UPR activation when α1ATZ accumulates in the ER could be explained in several possible ways. Recent studies have provided strong evidence that the UPR is initiated when BiP is titrated away from IRE1, PERK, and ATF-6 by misfolded proteins (4). It could therefore be hypothesized that the proximal transducers of the UPR are not activated by polymeric proteins because BiP is not titrated away by these proteins. However, here we found that antibody to BiP co-precipitated a polypeptide that co-migrates with α1ATZ only when α1ATZ gene expression is induced, and this co-precipitation looked almost identical to α1AT Saar even though BiP synthesis was only up-regulated in the latter (see Fig. 7c). By using detailed and quantitative sequential co-immunoprecipitation techniques and co-precipitation studies of sucrose density gradients under nondenaturing conditions, we have recently provided more definitive evidence that BiP binds to α1ATZ in transfected cell lines in the same way that it binds to α1AT Saar and α1AT SaarZ (19). Indeed, BiP and GRP94 can be found in heterogeneous soluble complexes together with multiple chaperones that are very similar when comparing cell lines that express α1ATZ, α1AT Saar, or α1AT SaarZ (19). These results militate against the possibility that α1ATZ fails to activate the UPR simply because it does not titrate BiP away from the proximal transducers of the UPR. Another explanation is that mutant α1ATZ in polymeric form does not titrate away as much BiP as the misfolded monomeric α1AT Saar and α1AT SaarZ do. Although our initial studies of this using several different concentrations of each of the antibodies (supplemental Fig. 2) do not support this notion, more detailed studies are needed to definitively address this possibility. Third, it is possible that polymers of α1ATZ titrate away BiP in specific sub-domains of the ER with the large bulk of IRE1, PERK, and ATF-6 molecules in the remainder of the ER still bound on their luminal domain by BiP molecules. It is also possible that BiP remains bound to IRE1, PERK, and ATF-6 when it is in soluble complexes with α1ATZ but not when in complex with α1AT Saar. An alternative explanation is that activation of the UPR requires dissociation of BiP under specific biochemical con-

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**FIGURE 11. Effect of ER retention of nonpolymerogenic mutant α1AT Saar on activation of the UPR, caspase-12, BAP31, and NFκB.**

- **a**. HepaTO/Saar cells were incubated in the presence or absence of dox for 10 days. The cells were then pulse-labeled, and the radiolabeled cell lysates were subjected to immunoprecipitation for α1AT (left) and BiP (right). b-d. HepaTO/M, HepaTO/Z, and HepaTO/Saar cell lines were incubated in the presence or absence of dox for 30 days. Cell lysates were then subjected to Western blot analysis for caspase-12 (b) or BAP31 (c1). Nuclear extracts were subjected to gel shift assay (d). C is the abbreviation for the positive control HeLa extract, and SS is the abbreviation for the supershift antibody, anti-p50.
ER Retention of Mutant α1ATZ Does Not Induce the UPR

ditions, such as raised intraluminal calcium concentrations, that are for some reason not found when insoluble polymers accumulate in the ER. We favor this explanation because it is most consistent with the two general concepts that have come from the studies here and elsewhere (19); ER retention of α1ATZ and α1AT Saar activates different signaling pathways; the only major difference in the disposition of α1ATZ and α1AT Saar is the presence in the ER of insoluble complexes in the case of α1ATZ.

Because previous studies had observed NFκB activation when proteins accumulate in the ER, the so-called ER overload pathway (6), we investigated the possibility that it was activated in response to the accumulation of α1ATZ in the ER. The results showed that NFκB was indeed activated in murine hepatoma cells when α1ATZ gene expression is induced. The fact that ER activation of α1ATZ activates NFκB but not the UPR provides strong corroborating evidence for the previously held contention that the ER overload pathway is distinct from the UPR (6). The ER overload pathway also has important implications for understanding target organ injury in α1AT-deficient patients. Through NFκB, accumulation of α1ATZ in the ER of liver cells and respiratory epithelial cells (28) could mediate the characteristic inflammation that occurs in the liver and lung of these patients, particularly neutrophil infiltration mediated by the NFκB target interleukin-8.

In a previous study we found that accumulation of α1ATZ in the ER was associated with mitochondrial injury and mitochondrial autophagy and that activation of caspase-3 could be detected in the liver of PiZ mice and affected human patients (11). The observation here that α1ATZ accumulation in the ER mediates cleavage of BAP31 suggests a possible mechanism for the effect on the mitochondria. By showing that ER accumulation of α1ATZ could also activate caspase-12 in the mouse and caspase-4 in the human, we now believe that caspases are activated in the liver in α1AT deficiency by both the ER and mitochondrial pathways. This also means that the ER-specific caspases can be activated in the absence of the UPR and, therein, that activation of the ER-specific caspase pathway is distinct from activation of the UPR. Recent studies have also shown that the UPR can be activated without accompanying caspase-4 or caspase-12 cleavage/activation (29). Even though accumulation of α1ATZ in the ER leads to activation of ER caspases, there is very little apoptosis detected in the liver of mouse models of and patients with α1AT deficiency (30). This may mean that the cellular response to ER accumulation of α1ATZ also induces the expression of caspase inhibitors that act downstream of caspase-3 activation, as described recently for the inhibitor or apoptosis protein XIAP2 (31). We have shown previously that several members of the heat shock protein family are up-regulated in cells from α1AT-deficient patients (32), and recent studies have shown that heat shock proteins can blunt apoptosis by a variety of mechanisms (33, 34). It is also worth noting that release of calcium by the ER has been implicated in the activation of NFκB by the ER overload pathway (6) and in the effect of ER stress on mitochondrial dysfunction/apoptosis (35), including the effects of BAP31 cleavage on mitochondria (10).

Finally it is notable that a distinct profile of signaling pathways is activated in response to accumulation of the polymeric mutant α1ATZ as compared with accumulation of the nonpolymeric mutants α1AT Saar and SaarZ. Accumulation of the Z mutant leads to activation of ER caspases, NFκB, and BAP31 but not the UPR, whereas accumulation of the Saar and SaarZ mutants leads to activation of the UPR and NFκB but not ER caspases or BAP31. At the very least, these data show that the mechanisms by which cells respond to the accumulation of mis-folded proteins in the ER may be substrate-specific. But we believe these data have even further implications. Although we do not know the full pathological significance of these response profiles, several aspects of the profile associated with the Z mutant are consistent with data on hepatocellular proliferation in the PiZ mouse model of α1AT deficiency (30). These data show that hepatocytes that accumulate α1ATZ, globule-containing hepatocytes, are relatively blocked in proliferation but do not have signs of cell death. However, the increase in the regenerative activity of the liver of the PiZ mouse is directly proportional to the number of globule-containing hepatocytes. We have concluded that the hepatocytes that accumulate α1ATZ are “sick but not dead” and responsible for transmitting regenerative signals in “trans” that are received by hepatocytes with lesser or no accumulation of α1ATZ, globule-devoid hepatocytes, that have a selective proliferative advantage. In fact, we have proposed that this is the basis of the predilection for hepatocellular carcinoma that is found in α1AT deficiency (36). Because activation of the UPR may lead to cell death, the lack of its activation in response to accumulation of α1ATZ may be part of the capacity of globule-containing hepatocytes to avoid cell death and chronically transmit regenerative signals in the liver. Activation of NFκB in these hepatocytes may also play a role in the pathobiology of hepatocellular carcinoma as it has been associated with inflammation-associated and chemically induced hepatic cancer (36).

REFERENCES

1. Perlmuter, D. H. (2002) J. Clin. Investig. 110, 1579–1583
2. Wu, Y., Whitman, I., Molmani, E., Moore, K., Hiperpenmueyer, P., and Perlmuter, D. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9014–9018
3. Kaufman, R. (2002) J. Investig. 110, 1389–1398
4. Ron, D. (2002) J. Investig. 110, 1383–1388
5. Zhang, K., and Kaufman, R. J. (2000) J. Biol. Chem. 275, 2935–29398
6. Pahl, H. L., Sester, M., Burgert, H.-G., and Bauserle, P. A. (1996) J. Cell. Biol. 132, 511–522
7. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. (2000) Nature 403, 98–103
8. Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., Manabe, T., Yamagishi, S., Bando, Y., Imai, I., Tsujimoto, Y., and Tohyama, M. (2004) J. Cell. Biol. 165, 347–356
9. Schamel, W. W. A., Kuppig, S., Becker, B., Gimborn, K., Hauri, H.-P., and Reth, M. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9861–9866
10. Breckenridge, D. G., Stojanovic, M., Marcellus, R. C., and Shore, G. C. (2003) J. Cell. Biol. 160, 1115–1127
11. Teckman, J. H., An, J. K., Blomenkamp, K., Schmidt, B., and Perlmuter, D. H. (2004) Am. J. Physiol. 286, G581–G582
12. Lin, L., Schmidt, B., Teckman, J., and Perlmuter, D. H. (2001) J. Biol. Chem. 276, 33893–33898
13. Kistner, A., Gossen, M., Zimmerman, F., Jerecic, J., Ullmer, C., Lubbert, H., and Bujard, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10933–10938
14. Carlson, J. A., Rogers, B. B., Sifers, R. N., Finegold, M. J., Clift, S. H., Francescato, J. D., Bullock, D. W., and Woo, S. L. C. (1989) J. Investig. 83, 1183–1190
15. Teckman, J. H., and Perlmuter, D. H. (2000) Am. J. Physiol. 279, G961–G974
16. Teckman, J. H., An, J. K., Loethen, S., and Perlmuter, D. H. (2002) Am. J. Physiol. 283, G1166–G1150
17. Qu, D., Teckman, J. H., Ornara, S., and Perlmuter, D. H. (1996) J. Biol. Chem. 271, 22791–22795
18. Zhang, Y., Nijbroek, G., Sullivan, M. L., McCracken, A. A., Watkins, S. C., Michaelis, S., and Brönsky, J. L. (2001) Mol. Biol. Cell 12, 1303–1314
19. Schmidt, B. Z., and Perlmuter, D. H. (2005) Am. J. Physiol. 289, G444–G455
20. Lee, K., Taraosh, W., Shen, X., Michahal, M., Prywes, R., Okada, T., Yoshida, H., Mori, K., and Kaufman, R. J. (2002) Genes Dev. 16, 452–466
21. Yue, X., Piao, J. H., Nakajima, A., Sekon-Komazawa, S., Kajima, Y., Nori, K., Yagitani, H., Okumura, K., Harding, H., and Nakano, H. (2005) J. Investig. 179, 33917–33925
22. Callon, M., Zeng, H., Urano, F., Till, J. N., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) Nature 415, 92–96
23. Zinszer, H., Kuroda, M., Wang, X. Z., Batcharova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) Genes Dev. 12, 982–995
24. Southwood, C. M., Garbenti, J., Jiang, W., and Gore, A. (2002) Neuron 36, 585–596
25. Oyadomari, S., Koizumi, A., Takeda, T., Gotoh, T., Akira, S., Araki, T., and Mori, M. (2002) J. Investig. 109, 525–532
26. Graham, K. S., Le, A., and Sifers, R. N. (1990) J. Biol. Chem. 265, 20463–20468
27. Lawless, M. W., Greene, C. M., Mulgrew, A., Taggart, C. C., O’Neill, S. J., and McElvany, N. G. (2004) J. Immunol. 172, 5722–5726
ER Retention of Mutant α1ATZ Does Not Induce the UPR

28. Hu, C., and Perlmutter, D. H. (2002) Am. J. Physiol. 282, L757–L765
29. Obeng, E. A., and Boise, L. H. (2005) J. Biol. Chem. 280, 29578–29587
30. Rudnick, D. A., Liao, Y., An, J. K., Muglia, L. J., Perlmutter, D. H., and Teckman, J. H. (2004) Hepatology 39, 1048–1055
31. Warnakulasuriyarachchi, D., Cerquozzi, S., Cheung, H., and Holcik, M. (2004) J. Biol. Chem. 279, 17148–17157
32. Perlmutter, D. H., Schlesinger, M. J., Pierce, J. A., Punsal, P. I., and Schwartz, A. L. (1989) J. Clin. Investig. 84, 1555–1561
33. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D., Mahboubi, A., Kuwana, T., Pankaj, T., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000) Nat. Cell Biol. 2, 469–475
34. Takayama, S., Reed, J. C., and Homma, S. (2003) Oncogene 22, 9041–9047
35. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) Science 300, 135–141
36. Rudnick, D. A., and Perlmutter, D. H. (2005) Hepatology 42, 514–521