# Synthesis of Stress Proteins Is Increased in Individuals with Homozygous PiZZ $\alpha_1$-Antitrypsin Deficiency and Liver Disease

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## Abstract

**Individuals who are homozygous for the protease inhibitor phenotype Z (PiZ) genetic variant of $\alpha_1$-antitrypsin ($\alpha_1$-AT) have reduced plasma concentrations of $\alpha_1$-AT, and are susceptible to premature development of pulmonary emphysema. A subset of this population develops chronic liver disease. The reduction in plasma concentrations of $\alpha_1$-AT results from a selective defect in secretion as the abnormal PiZ $\alpha_1$-AT protein accumulates within the cell. It has recently been shown in several experimental systems that the heat shock/stress response, a response characterized by the synthesis of a family of highly evolutionarily conserved proteins during thermal or chemical stress, may also be activated by the presence of abnormal proteins within the cell. Therefore, we predicted that the heat shock/stress response would be induced in the absence of thermal or chemical stress in $\alpha_1$-AT-synthesizing cells of PiZZ individuals. In the following study, however, we show that net synthesis of proteins in the heat shock/stress gene family (SP90, SP70, ubiquitin) is increased only in a subset of the population, PiZZ individuals with liver disease. It is not significantly increased in PiZZ individuals with emphysema or in those without apparent tissue injury. Net synthesis of stress proteins is not increased in individuals with another variant of the $\alpha_1$-AT gene (PiS $\alpha_1$-AT) and is not increased in individuals with severe liver disease but a normal $\alpha_1$-AT haplotype (PiM $\alpha_1$-AT). These results demonstrate that the synthesis of stress proteins is increased in a subset of individuals with homozygous PiZZ $\alpha_1$-AT deficiency, those also having liver disease.**

## Introduction

Homozygous PiZZ $\alpha_1$-antitrypsin ($\alpha_1$-AT)[1] deficiency is a common genetic disorder resulting in low plasma concentrations of functionally active $\alpha_1$-AT (reviewed in references 1 and 2). This deficiency affects approximately 1 in 1,600 live births (3, 4) and is associated with premature development of pulmonary emphysema, chronic liver disease, and hepatocellular carcinoma (5, 6). $\alpha_1$-AT deficiency is also the most common metabolic disease for which individuals currently undergo orthotopic liver transplantation (7).

The association of this deficiency with liver disease has been difficult to explain. It has been even more difficult to explain the fact that only 12–15% of the PiZZ $\alpha_1$-AT-deficient population develop significant liver injury (6). According to one theory, accumulation of $\alpha_1$-AT in endoplasmic reticulum of liver cells is directly related to liver injury (2). Evidence supporting the "accumulation theory" of liver injury includes the absence of histologic changes in liver in rare protease inhibitor (Pi) null variants, allelic variants in which $\alpha_1$-AT is not synthesized. Moreover, liver disease has developed in several litters of transgenic mice carrying the mutant allele of the human $\alpha_1$-AT gene (8, 9).

The pathophysiology of lung disease is thought to involve a decrease in plasma concentrations of the major anti-elastase, $\alpha_1$-AT, which permits elastolytic attack on the lung (reviewed in reference 10). Environmental factors, such as cigarette smoking which may oxidatively inactivate $\alpha_1$-AT, may also contribute to the development of emphysema. Nevertheless, there is still variability in the incidence and severity of destructive lung disease even when environmental factors are taken into consideration.

The molecular/cellular pathogenesis of homozygous PiZZ $\alpha_1$-AT deficiency has been studied extensively. It is now known that there is a selective defect in secretion of $\alpha_1$-AT in human blood monocytes from PiZZ individuals (11, 12), in *Xenopus* oocytes injected with RNA from liver of PiZZ individuals (11, 13, 14), and in cell lines transfected with the mutant PiZ allele (15–17). In these circumstances the abnormal protein accumulates within the cell. Electron microscopic analyses (18) of liver from these individuals suggest that the abnormal protein accumulates within the endoplasmic reticulum (ER). These studies, therefore, suggest that the defect in PiZZ cells affects transport of newly synthesized $\alpha_1$-AT from the ER to the Golgi complex. Several recent studies using site-directed mutagenesis (15–17, 19) have shown that the substitution of lysine for glutamate at residue 342 in the $\alpha_1$-AT coding sequence of the PiZ allele (20–24) is enough to cause a selective defect in secretion. These studies have also shown that the disruption of a salt bridge between $glu^{342}$ and $lys^{290}$, as predicted from x-ray crystallographic determinations (25), could not entirely account for the defect in secretion. Thus, it has been suggested that the PiZ substitution results in an abnormality in folding, or tertiary structure, of the newly synthesized $\alpha_1$-AT protein, or a change in specific protein–protein interactions involved in transport or retention within the secretory pathway.

The stress response, which is characterized by a marked increase in production of a family of highly evolutionarily conserved and closely related proteins during thermal or chemical stress, may also be activated by the presence of ab-

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1. *Abbreviations used in this paper:* $\alpha_1$-AT, $\alpha_1$-antitrypsin; ER, endoplasmic reticulum; Pi, protease inhibitor.

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normally folded proteins within the cell (26–28). With the same considerations in mind, we examined the possibility that the stress proteins are induced under resting conditions in cells of PiZZ individuals inside which the abnormal $\alpha_1$-AT molecule accumulates. Since we have previously shown that $\alpha_1$-AT accumulates within monocytes from PiZZ individuals as well as within *Xenopus* oocytes injected with PiZZ liver RNA (11), the present study was conducted with human blood monocytes and human liver RNA. Studies of monocytes also allowed us to examine the relationship between synthesis of stress proteins and $\alpha_1$-AT deficiency in each of its clinical phenotypes (lung disease, liver disease, no apparent disease).

## Methods

*Patients.* Peripheral blood monocytes were isolated from 24 normal PiMM donors, 7 PiZZ individuals with liver disease, 6 PiZZ individuals with emphysema, 5 PiZZ individuals without evidence of liver or lung involvement, 5 PiMM individuals with severe liver disease, and 2 normal healthy individuals with the PiSS $\alpha_1$-AT phenotype. Ages ranged from 3 yr and 7 mo to 52 yr. There were no significant age-related differences in synthesis of stress proteins in the normal control subjects. For each experiment, monocytes from an experimental subject (PiZZ individual, PiSS individual, or PiMM individual with severe liver disease) were studied simultaneously with monocytes from a concurrent normal healthy PiMM control. Normal subjects had normal serum levels of $\alpha_1$-AT and phenotype PiMM determined by isoelectric focusing. Diagnosis of PiZZ $\alpha_1$-AT deficiency was established by determination of serum levels by rocket immunoelectrophoresis, Pi phenotype by isoelectric focusing, and family studies. The diagnoses of emphysema and liver disease were established by clinical signs and symptoms, serum transaminases, liver function studies, pulmonary function studies, and liver biopsy in clinically indicated circumstances. The five individuals with homozygous PiZZ $\alpha_1$-AT deficiency without clinical or biochemical evidence of liver or lung disease included the following: a 14-yr-old boy and a 5-yr-old boy each of whom had elevated serum bilirubin only in the first year of life; two siblings of PiZZ individuals who had liver disease; and a 38-yr-old sibling of two PiZZ individuals both of whom had emphysema.

RNA was isolated from the native livers of six PiZZ, five PiMZ, and seven PiMM who underwent orthotopic liver transplantation. The PiMZ or PiMM individuals with liver disease had diagnoses of idiopathic postnecrotic cirrhosis or chronic active hepatitis with cirrhosis on the basis of clinical and histological criteria.

*Materials.* DME and DME lacking methionine were purchased from Gibco Laboratories, Grand Island, NY. HBSS and Medium 199 (M199) were purchased from Microbiological Associates, Walkersville, MD. Fetal calf serum, L-glutamine, and penicillin-streptomycin were from Flow Laboratories, Inc., McLean, VA. [$^{35}$S]Methionine were purchased from ICN Radiochemicals, Costa Mesa, CA; [$^{32}$P]deoxycytidine triphosphate (specific radioactivity ~ 3,000 Ci/mmol) was obtained from New England Nuclear, Boston, MA; and [$^{14}$C]methylated protein standards were from Amersham Corp., Arlington Heights, IL. Other reagents included IgG-Sorb from Enzyme Center, Cambridge, MA and guanidine isothiocyanate from Fluka AG, Buchs, Switzerland. Rabbit anti-human $\alpha_1$-AT was from Dako Corp., Santa Barbara, CA, and goat anti-human factor B was purchased from Atlantic Antibodies, Scarborough, ME. Antibodies to SP90, SP70, and unconjugated ubiquitin have been previously described (29, 30). Endotoxin (LPS) preparations extracted from *Escherichia coli* serotype 0111.B4 by Westphal phenolic extraction were purchased from Sigma Chemical Co., St. Louis, MO. *E. coli* 0113 endotoxin (LPS) from Associates of Cape Cod, Inc., Woods Hole, MA, was also used.

*Cell culture.* Confluent monolayers of human peripheral blood monocytes were established by adherence of dextran-purified leukocytes on charged tissue culture plates and dishes (Primaria, Becton-Dickinson Labware, Lincoln Park, NJ) by a previously described protocol (31). In some experiments, monolayers of monocytes were "stressed" by incubation at 42°C for 2 h. This condition was shown to cause marked increases in synthesis of SP70 and SP90 and increases in steady-state levels of polyubiquitin mRNA in normal PiMM monocytes. Incubation of normal monocytes at 42°C for longer time intervals did not result in further increases in polyubiquitin mRNA levels (data not shown).

*Metabolic labeling.* Confluent monolayers were rinsed and incubated for 30 min at 37°C in the presence of methionine-free medium containing [$^{35}$S]methionine, 500 $\mu$Ci/ml. Radiolabeled proteins were detected in the cell lysate alone. Since this method does not account for amino acid pool size or the small amount of catabolism that might occur within 30 min, we refer to the results as net synthesis throughout the paper. Methods for solubilization of cells and clarification of cell lysates after labeling have been described (32). Total protein synthesis was estimated by TCA precipitation of aliquots of cell lysates and culture fluid (33).

*Immunoprecipitation and analytical gel electrophoresis.* Aliquots of cell lysates were incubated overnight at 4°C in 1% Triton X-100/1.0% SDS/0.5% deoxycholic acid, with excess antibody. Immune complexes were precipitated with excess formalin-fixed staphylococci-bearing protein A, washed, released by boiling in sample buffer, and applied to 9.0% SDS-PAGE under reducing conditions (34). $^{14}$C-methylated molecular size markers (200,000, 95,000, 68,000, 46,000, 30,000, and 17,000 mol wt) were incubated on all gels. After electrophoresis, gels were stained in Coomassie brilliant blue, destained, impregnated with 2,5-diphenyloxazole (EN$^3$HANCE, New England Nuclear), and dried for fluorography on XAR x-ray film (Eastman Kodak Co., Rochester, NY). Laser densitometer 2222 ultrascan XL from LKB Instruments, Inc., Houston, TX, was used for scanning of fluorograms.

*Detection of RNA by RNA blot analysis.* Total cellular RNA was isolated from adherent monolayers of monocytes or liver by guanidine isothiocyanate extraction and ethanol precipitation (35). RNA was subjected to agarose-formaldehyde gel electrophoresis and transferred to nitrocellulose filters (36). Filters were then hybridized with $^{32}$P-labeled cDNA specific for human $\alpha_1$-AT (31), ubiquitin (37), or SP90 (kindly provided by N. Rebbe, St. Louis, MO).

## Results

*Net synthesis of stress proteins is increased in monocytes from PiZZ individuals with liver disease.* Separate monolayers of peripheral blood monocytes from a healthy PiMM donor and a PiZZ individual with liver disease were incubated for 30 min at 37°C with [$^{35}$S]methionine (Fig. 1). Cell lysates were subjected to immunoprecipitation with polyclonal antibodies to SP90, SP70 (29), unconjugated ubiquitin (36), and complement protein factor B. The antibodies to SP90 and SP70 recognize the corresponding stress proteins and their cognates (including glucose-regulated proteins) (29). Synthesis of SP90 and SP70 is easily demonstrated in the PiZZ monocytes but not detected in the PiMM monocytes under control culture conditions (*Control* in Fig. 1, *a* and *b*). These differences are not due to inability to detect the stress proteins in the PiMM monocytes since radiolabeled SP70 and SP90 are detected in PiMM monocytes incubated at 42°C (stress) for 2 h before labeling (Fig. 1, *a* and *b*). In some PiZZ monocytes, antibody to SP70 immunoprecipitates a radiolabeled polypeptide of ~ 50 kD in apparent size (Fig. 1 *b*), whereas in others it immunoprecipitates a radiolabeled polypeptide of ~ 70 kD (data not shown). Polypeptides apparently smaller than 70 kD have been immunoprecipitated with antibody to SP70 in other mammalian cell culture systems (38), and are thought to be degradation products. In the circumstances reported here, it is

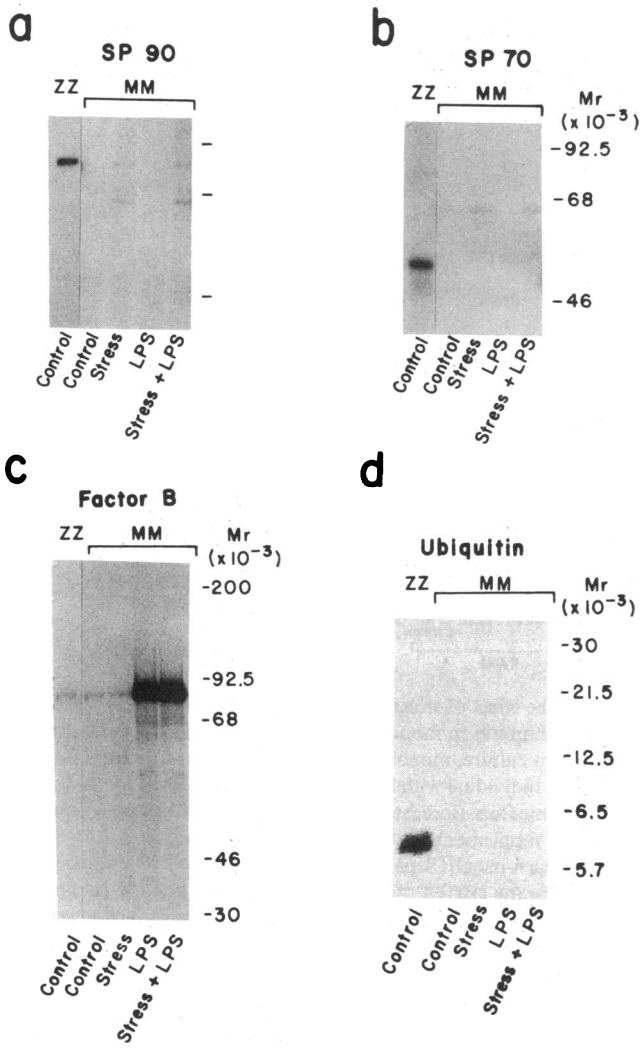


*Figure 1.* Net synthesis of SP90, SP70, and ubiquitin in human monocytes. After 24 h in culture, monocytes from a PiZZ individual with liver disease and from a normal PiMM donor (as indicated at the top of each panel) were incubated for 24 h in serum-free medium (indicated as *Control* at the bottom of each panel). Separate monolayers of monocytes from the PiMM individual were incubated for 24 h in medium supplemented with endotoxin 100 ng/ml (indicated as *LPS* at the bottom of each panel). Still other monolayers from the PiMM individual were incubated for last 2 h of the incubation period at 42°C (indicated as *Stress* or *Stress + LPS* at the bottom of each panel). All these monocytes were then labeled with [$^{35}$S]-methionine for 30 min. Cell lysates were subjected to immunoprecipitation with the specified antibodies before SDS-PAGE/fluorography. Molecular mass markers are shown at the right margin. In *a* and *b* the same fluorograms were exposed to x-ray film for 1 d to show the lane from PiZZ monocytes and 12 d to show the lanes from PiMM monocytes. The mobility of purified unconjugated ubiquitin in the same gel system (15% SDS-PAGE) was identical to the radiolabeled polypeptide shown in *d* (data not shown).

also possible that the ~ 50-kD polypeptide represents $\alpha_1$-AT in complex with SP70. However, extensive investigation will be necessary to prove or exclude this possibility. Endotoxin (LPS), which increases synthesis of $\alpha_1$-AT in PiMM and PiZZ monocytes, but only increases intracellular accumulation of $\alpha_1$-AT in PiZZ monocytes (31), does not affect the net synthesis of SP90 or SP70 in PiMM monocytes (Fig. 1, *a* and *b*).

Thus, there is a marked increase in net synthesis of stress proteins in PiZZ monocytes demonstrated by immunoprecipitation of radiolabeled cell lysates. The magnitude of this increase is > 15-fold on average (see Fig. 2) but not great enough to be detected by subjecting the total radiolabeled cell protein directly to gel electrophoresis followed by fluorography (data not shown).

The marked difference in net synthesis of SP90 and SP70 in PiZZ as compared with PiMM monocytes is not due to differences in the amount of radiolabeled proteins subjected to analysis since there is an equivalent amount of newly synthesized factor B in control PiZZ monocytes, and control and stressed PiMM monocytes (Fig. 1 *c*). Furthermore, factor B expression is inducible in these cells as shown by an increase in its synthesis mediated by LPS (Fig. 1 *c* [39]).

We next examined the net synthesis of another stress protein, ubiquitin, in the same cells (Fig. 1 *d*). Net synthesis of ubiquitin is easily demonstrated in control PiZZ monocytes but not detected in this experiment in control, stress-induced, or LPS-induced PiMM monocytes. It is barely detectable only in stress-induced PiMM monocytes in other experiments (see Fig. 3 below).

Net synthesis of SP90 was examined in monocytes from PiZZ individuals with liver disease, PiZZ individuals with emphysema, several asymptomatic PiZZ individuals, PiMM individuals with severe liver disease, or asymptomatic PiSS individuals (Fig. 2). For each of these individuals net synthesis of SP90 is compared with net synthesis of SP90 in monocytes from a concurrent normal PiMM individual after stress and is expressed as fold increase over this control. There is a marked increase in net synthesis of SP90 in monocytes of PiZZ individuals with liver disease. The mean increase in this group is greater than 15-fold that detected in cells from normal individuals. The individual with the lowest relative increase is 2.6-fold higher than her control. Relative levels of SP90 synthesis did not correlate with the severity of liver disease or any other clinical characteristics. There is a smaller increase in SP90 in cells from individuals with emphysema. The mean value in the emphysema group is greatly affected by results from one individual in whom we have subsequently discovered biochemical evidence for liver injury (denoted by Δ in Fig. 2). Net synthesis of SP90 in asymptomatic PiZZ individuals is even lower than in PiZZ individuals with emphysema. There is no increase in the net synthesis of SP90 in five PiMM individuals with severe liver disease or in two individuals homozygous for the PiS $\alpha_1$-AT allele. PiS $\alpha_1$-AT is an allelic variant which is not associated with disease and not associated with intracellular $\alpha_1$-AT accumulation (2). A single nucleotide substitution in the PiS allele may result in a decrease in $\alpha_1$-AT mRNA or protein stability but only 40–50% reduction in plasma concentrations of $\alpha_1$-AT.

We also examined net synthesis of SP90 in lymphocytes, cells which do not synthesize $\alpha_1$-AT (40). In contrast to the marked differences observed in monocytes, there is no difference in SP90 synthesis in lymphocytes from PiZZ individuals with liver disease as compared to lymphocytes from normal PiMM individuals (data not shown). This result suggests that the increase in net synthesis of SP90 in PiZZ individuals is confined to cells which synthesize $\alpha_1$-AT.

*Effect of stress and LPS on net synthesis of stress proteins in normal and deficient monocytes.* In order to understand further the stress response in $\alpha_1$-AT deficiency we studied the

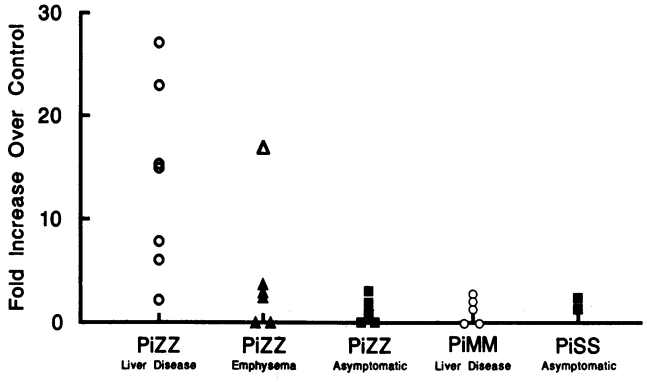


*Figure 2.* Net synthesis of SP90 in monocytes from PiZZ, PiMM, and PiSS individuals with different clinical manifestations. Monocytes were incubated under control conditions or exposed to 42°C for 2 h (as described in legend to Fig. 1) and then labeled with [$^{35}$S]-methionine for 30 min. Cell lysates were subjected to immunoprecipitation with antibody to SP90, followed by SDS/PAGE/fluorography. Synthesis of SP90 was then determined by densitometric scanning of fluorograms using an LKB laser densitometer 2222 Ultrascan XL. In each case net synthesis of SP90 after stress (heat shock) was compared with net synthesis of SP90 in monocytes from a concurrent normal PiMM donor also subjected to stress (heat shock). Results are expressed along the vertical axis as fold increase over control (control represents concurrent PiMM donor for each patient included). In each case there was equivalent net synthesis of a constitutive protein, factor B. It was possible to make this quantitative comparison of net SP90 synthesis in monocytes from different types of patients under stress (heat shock) conditions because, in the assay system used for these experiments, synthesis of SP90 is undetectable in normal PiMM monocytes in the absence of stress (see Fig. 1). Heat shock was chosen because it induces the most abundant and well-characterized effect on stress proteins. Individuals with liver disease are denoted by open symbols.

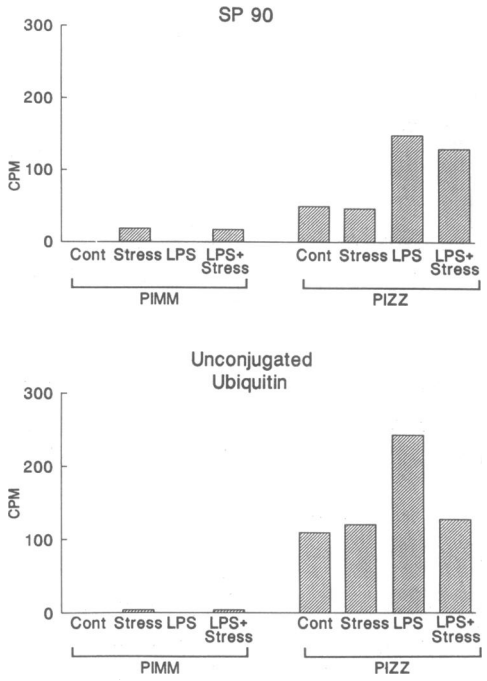


*Figure 3.* The effect of stress and endotoxin (LPS) on net synthesis of SP90 and ubiquitin in monocytes from PiMM and PiZZ individuals. After 24 h in culture, monocytes from a normal PiMM individual and a PiZZ individual with liver disease were incubated for 24 h in serum-free medium (indicated as *Cont* at the bottom of each panel), or medium supplemented with LPS, 100 ng/ml (indicated *LPS* at the bottom of each panel). Separate monolayers from each donor were incubated for the last 2 h of the incubation period at 42°C (indicated as *Stress* or *LPS* + *Stress* at the bottom of each panel). These were entirely different experiments and different donors from those shown in Fig. 1. Net synthesis of SP90 and ubiquitin was determined by labeling with [$^{35}$S]methionine, immunoprecipitation, and SDS-PAGE fluorography. Radiolabeled polypeptides corresponding to each protein were excised from the gels, solubilized overnight in 15% hydrogen peroxide at 50°C and subjected to direct scintillation counting. This experiment is representative of three experiments with separate donors.

effect of stress and/or LPS on net synthesis of SP90 and ubiquitin in monocytes (Fig. 3). Separate monolayers of monocytes from PiMM and PiZZ individuals were incubated in serum-free control medium (*Cont*) or medium supplemented with LPS (*LPS*). In each case the monolayers were incubated for an additional 2 h at 37°C or 42°C (*Stress*). Cells were then labeled with [$^{35}$S]methionine and newly synthesized SP90 and ubiquitin demonstrated by immunoprecipitation, SDS-PAGE, and fluorography. Radiolabeled bands corresponding to each protein were excised from the gel, solubilized, and subjected to scintillation counting. In PiMM monocytes, stress, but not LPS, induces net synthesis of SP90. Net synthesis of unconjugated ubiquitin is barely detectable in PiMM monocytes only after stress. In monocytes from a PiZZ individual with liver disease, net synthesis of SP90 and ubiquitin is already elevated in the control condition. It is further increased by LPS, but not by stress. As mentioned above, LPS is known to increase synthesis of $\alpha_1$-AT in PiMM and PiZZ monocytes but only increases intracellular accumulation of $\alpha_1$-AT in PiZZ monocytes (31). This result suggests that stress protein synthesis is related to accumulation of the abnormal protein. In monocytes from PiZZ individuals with emphysema alone or without apparent tissue injury, net synthesis of SP90 is inducible with stress but not with LPS (data not shown). That is, net synthesis of heat shock proteins in these latter individuals is more like that of normal PiMM individuals. In the single PiZZ individual with emphysema, biochemical evidence of liver disease and high levels of SP90 synthesis (Fig. 2, △), induction of SP90 is similar to that of PiZZ individuals with liver disease alone (data not shown).

LPS and stress appear to have counterregulatory effects on net synthesis of ubiquitin in monocytes from PiZZ individuals with liver disease. Similar counterregulatory effects have been observed for different types of stress in other mammalian cell culture systems (41) but the mechanisms are still not understood.

*Steady-state levels of ubiquitin mRNA are increased in liver of PiZZ individuals.* An increase in expression of the genes encoding stress proteins is also evident in liver of PiZZ individuals as shown by steady-state levels of ubiquitin mRNA in native livers of three PiZZ, two PiMZ, and two PiMM individuals who underwent liver transplantation (Fig. 4, *upper panel*). All seven individuals had severe liver disease. The PiMZ and PiMM individuals carried diagnoses of idiopathic postnecrotic cirrhosis or chronic active hepatitis with cirrhosis. Levels of ubiquitin mRNA are higher in all three PiZZ livers. There are no significant differences in the level of $\alpha_1$-AT mRNA in these

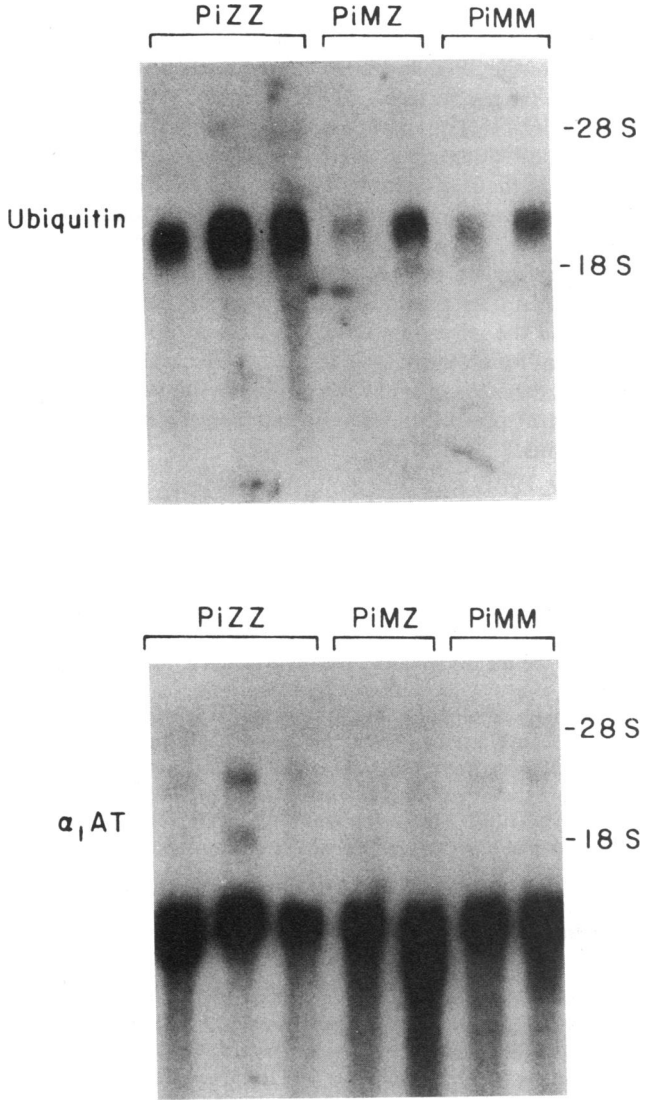


*Figure 4.* Steady-state levels of ubiquitin mRNA in livers from PiZZ, PiMZ, and PiMM individuals. Total cellular RNA was subjected to RNA blot analysis with $^{32}$P-labeled polyubiquitin cDNA and $\alpha_1$-AT cDNA. Filters were then washed extensively and exposed to x-ray film for autoradiography. Similar amounts of ethidium bromide–stained 28S and 18S RNA were visualized in each lane. DNA size markers are indicated at the right margin.

livers (*lower panel*). In order to determine the statistical significance of this difference a number of additional patients were studied. Steady-state levels of ubiquitin mRNA were determined in a total of six PiZZ, five PiMZ, and seven PiMM individuals with severe liver disease. The results of densitometric scanning of RNA blots is shown in Fig. 5. Ubiquitin RNA levels are approximately threefold higher ($P < 0.005$) in liver of PiZZ than that in PiMZ and in PiMM individuals. These blots were also subjected to hybridization with a human SP90 cDNA probe. Steady-state levels of SP90 mRNA were also significantly increased in liver of PiZZ individuals (data not shown). These data, therefore, suggest that $\alpha_1$-AT deficiency is associated with an increase in expression of stress proteins above and beyond that which might occur nonspecifically during liver injury.

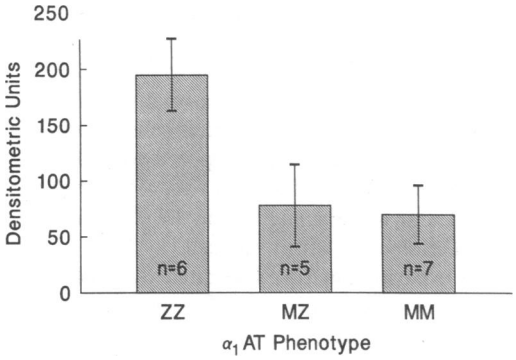


*Figure 5.* Relative levels of ubiquitin mRNA in livers from PiZZ, PiMZ, and PiMM individuals. Total cellular RNA from a total of six PiZZ, five PiMZ, and seven PiMM individuals (including those in Fig. 3) was subjected to a single RNA blot analysis with $^{32}$P-labeled polyubiquitin cDNA probe. The intensity of the 2.4-kb ubiquitin mRNA was determined by densitometric scanning of the autoradiogram and is expressed as relative densitometric units. Each hatched bar represents a mean and each bracket represents 1 SD. Values for PiZZ individuals were compared separately to those for PiMZ and PiMM individuals by Student's $t$ test. In each case the difference is statistically significant ($P < 0.005$).

## Discussion

These results indicate that the physiologic stress response is induced in the absence of thermal or chemical stress in $\alpha_1$-AT–synthesizing cells of PiZZ individuals with liver disease. In normal cells the stress response is only induced by experimentally induced thermal or chemical signals. The "uninduced" stress response is specific for PiZZ individuals with liver disease: it is not found in asymptomatic PiZZ individuals or individuals with the PiS $\alpha_1$-AT allele. The uninduced stress response cannot be attributed to a nonspecific effect of tissue injury or inflammation: net synthesis of stress proteins is not increased in PiMM individuals with severe liver disease.

Several lines of evidence suggest that the stress response is related to the intracellular accumulation of the mutant $\alpha_1$-AT protein: there is a reduction in plasma concentrations of $\alpha_1$-AT in individuals homozygous for the PiZ and PiS allele but intracellular accumulation of the abnormal gene product is only observed with the PiZ allele; the increase in net synthesis of stress proteins is confined to $\alpha_1$-AT–synthesizing cells of PiZZ individuals with liver disease and is exaggerated by regulatory factors that cause greater intracellular accumulation of $\alpha_1$-AT in cells of PiZZ individuals (e.g. LPS [31]). Nevertheless, further studies are necessary to establish a causal relationship.

In this study we have only examined PiZZ individuals with obvious liver disease. Therefore, it is not possible to draw any conclusions about the occasional PiZZ individual with inapparent liver injury, i.e., the PiZZ individual found to have cirrhosis or hepatoma in the absence of clinical or biochemical abnormalities (6). There are several possible explanations for induction of stress genes to affect deficient individuals with liver disease but not deficient individuals with lung disease or asymptomatic deficient individuals. Deficient individuals with liver disease may have a second, unlinked genetic trait that determines a slower rate of removal, or degradation, of accumulated $\alpha_1$-AT than PiZZ individuals who develop emphysema or PiZZ individuals who do not develop tissue injury.

Alternatively this trait may be associated with an intrinsic difference in the function and/or activation of the stress protein system. Such a trait would only be manifest in the PiZZ individual, an individual in whose cells an abnormal protein continuously accumulates. It is also possible that cells which synthesize $\alpha_1$-AT in these individuals are exposed to higher, or more sustained, concentrations of factors which enhance the expression of $\alpha_1$-AT and, therein, accentuate the accumulation of abnormal $\alpha_1$-AT molecules within the cell.

Three types of experiments have indicated that the stress response is induced by the presence of abnormally folded proteins within the cell. There is a marked increase of stress proteins within cells which accumulate abnormal proteins during incubation with amino acid analogues (26). Expression of stress protein SP70 is induced in frog oocytes by microinjection of protein which has been denatured before injection, but not by the same protein introduced in native form (27). A recent study by Kozutsumi et al. (28) showed that mutant forms of influenza virus hemagglutinin, whose transport from the endoplasmic reticulum was blocked, induced the expression of several members of the stress protein family. There is also a growing body of evidence that one function of stress proteins is the biochemical monitoring of proper protein folding within the cell. For instance, several recent studies have suggested that SP70 is responsible for the unfolding of proteins during translocation across microsomal (42) and mitochondrial membranes (43). One SP70-like protein, the immunoglobulin heavy chain binding protein (BiP), is now known to reside in the ER (44). In this compartment the protein transiently binds abnormal or incorrectly assembled proteins until they have achieved a mature folded state (reviewed in reference 45). Another stress protein, ubiquitin, is conjugated to proteins destined for degradation within the cell (reviewed in references 41 and 46). Thus, the function of stress proteins may be especially relevant to genetic defects in which abnormally folded proteins accumulate within the cell.

In experimental systems induction of the stress response by abnormally folded proteins is thought to be mediated by a complex of the abnormally folded protein and a stress protein within a specific intracellular compartment (28). In the case of $\alpha_1$-AT deficiency, then, one might envisage a genetically determined abnormality in folding which exposes a relatively hydrophobic epitope on $\alpha_1$-AT after the nascent protein is translocated into the lumen of the endoplasmic reticulum and results in its binding by a stress-related protein residing in that compartment. This type of interaction would explain our previous observation that increases in synthesis of $\alpha_1$-AT result in greater intracellular accumulation of the mutant $\alpha_1$-AT protein in cells of PiZZ individuals (31, 47).

One alternative explanation for induction of stress proteins in $\alpha_1$-AT deficiency must be considered. The substitution of lysine for glutamate at residue 342 in the PiZ $\alpha_1$-AT allele could lead to an abnormality in folding of the nascent protein that not only affects transport within the ER but also reduces the efficiency of translocation into the lumen of ER. Abnormally folded $\alpha_1$-AT might then also accumulate in the cytosol and induce stress proteins such as SP70, SP90, and ubiquitin. However, this explanation is less likely since previous studies have indicated that the efficiency of translocation into microsomal membranes in vitro is similar for PiM and PiZ $\alpha_1$-AT (14, 48).

Two other issues raised by these data will require further study. First, since antibodies to SP70 and SP90 used in these experiments recognize a number of highly homologous proteins within the SP70 and SP90 family, it will be necessary to determine the specific protein(s) induced and the specific protein(s) which bind(s) mutant $\alpha_1$-AT. Secondly, if antibodies to SP70 and SP90 are actually recognizing a cognate which resides within the endoplasmic reticulum of PiZZ cells, then it will be necessary to determine why ubiquitin, thought to be localized to the cytosolic compartment, is also induced.

Taken together, these data demonstrate a specific association between liver disease in homozygous PiZZ $\alpha_1$-AT deficiency and the stress response. The data do not address the mechanism for defective secretion of $\alpha_1$-AT in the PiZ genetic variant or the mechanism for liver injury in this variant, but do raise several possibilities for further investigation of both mechanisms.

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## References

1. Gadek, J. E., and R. G. Crystal. 1983. $\alpha_1$-Antitrypsin deficiency. *In* The Metabolic Basis of Inherited Disease. 5th edition. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill Book Co., Inc., New York. 1450–1467.

2. Carrell, R. W. 1986. $\alpha_1$-Antitrypsin: molecular pathology, leukocytes and tissue damage. *J. Clin. Invest.* 77:1427–1431.

3. Sveger, T. 1976. Liver disease in $\alpha_1$-antitrypsin deficiency detected by screening of 200,000 infants. *N. Engl. J. Med.* 294:1316–1321.

4. Silverman, E. K., J. P. Miletich, J. A. Pierce, L. A. Sherman, G. J. Broze, and E. J. Campbell. 1989. Alpha-1-antitrypsin deficiency: prevalence estimation from direct population screening. *Am. Rev. Respir. Dis.* In press.

5. Sveger, T. 1988. The natural history of liver disease in $\alpha_1$-antitrypsin deficient children. *Acta Paediatr. Scand.* 77:847–851.

6. Eriksson, S., J. Carlson, and R. Velez. 1986. Risk of cirrhosis and primary liver cancer in alpha-1-antitrypsin deficiency. *N. Engl. J. Med.* 314:736–739.

7. Gartner, J. C., B. J. Zitelli, J. J. Malatack, B. W. Shaw, S. Iwatsuki, and T. E. Starzl. 1984. Orthotopic liver transplantation in children: two-year experience with 47 patients. *Pediatrics.* 74:140–145.

8. Dycaico, M. J., S. G. N. Grant, K. Felts, W. S. Nichols, S. A. Geller, J. H. Hager, A. J. Pollard, S. W. Kohler, H. P. Short, F. R. Jirik, D. Hanahan, and J. A. Sorge. 1988. Neonatal hepatitis induced by $\alpha_1$-antitrypsin: a transgenic mouse model. *Science (Wash. DC).* 242:1409–1412.

9. Carlson, J. A., B. B. Rogers, R. N. Sifers, M. J. Finegold, S. M. Clift, F. J. DeMayo, D. W. Bullock, and S. L. C. Woo. 1989. Accumulation of PiZ $\alpha_1$-antitrypsin causes liver damage in transgenic mice. *J. Clin. Invest.* 83:1183–1190.

10. Gadek, J. E., G. A. Fells, R. L. Zimmerman, S. I. Rennard, and R. G. Crystal. 1981. Antielastases of the human alveolar structure: implications for the protease-antiprotease theory of emphysema. *J. Clin. Invest.* 68:889–898.

11. Perlmutter, D. H., R. M. Kay, F. S. Cole, T. H. Rossing, D. H. Van Thiel, and H. R. Colten. 1985. The cellular defect in $\alpha_1$-proteinase inhibitor deficiency is expressed in human monocytes and in *Xenopus* oocytes injected with human liver mRNA. *Proc. Natl. Acad. Sci. USA.* 82:6918–6921.

12. Mornex, J.-F., A. Chytil-Weir, Y. Martinet, M. Courtney, J. P. LeCocq, and R. G. Crystal. 1986. Expression of the alpha-1-antitrypsin gene in mononuclear phagocytes of normal and alpha-1-antitrypsin-deficient individuals. *J. Clin. Invest.* 77:1952–1961.

13. Foreman, R. C., J. D. Judah, and A. Colman. 1984. *Xenopus* oocytes can synthesise but do not secrete the Z variant of human $\alpha$-1-antitrypsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 168:84–88.

14. Verbanac, K. M., and E. C. Heath. 1986. Biosynthesis, processing and secretion of M and Z variant human $\alpha$-1-antitrypsin. *J. Biol. Chem.* 261:9979–9989.

15. Brantly, M., M. Courtney, and R. G. Crystal. 1988. Repair of the secretion defect in the Z form of $\alpha_1$-antitrypsin by addition of a second mutation. *Science (Wash. DC).* 242:1700–1702.

16. McCracken, A. A., K. B. Kruse, and J. L. Brown. 1989. Molecular basis for defecive secretion of the Z variant of human alpha-1-proteinase inhibitor: secretion of variants having altered potential for salt bridge formation between amino acids 240 and 342. *Mol. Cell. Biol.* 9:1406–1414.

17. Sifers, R. N., C. P. Hardick, and S. L. C. Woo. 1989. Disruption of the 240–342 salt bridge is not responsible for the secretory defect of the PiZ $\alpha_1$-antitrypsin variant. *J. Biol. Chem.* 264:2997–3002.

18. Feldmann, G., J. Bignon, P. Chahinian, C. Degott, and J.-P. Benhamou. 1974. Hepatocyte ultrastructural charges in $\alpha$-1-antitrypsin deficiency. *Gastroenterology.* 67:1214–1224.

19. Foreman, R. C. 1987. Disruption of the lys 290- glu 342 salt bridge in human $\alpha_1$-antitrypsin does not prevent its synthesis and secretion. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 216:79–82.

20. Jeppsson, J.-O. 1976. Amino acid substitution Glu-Lys in $\alpha_1$-antitrypsin PiZ. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 65:195–197.

21. Yoshida, L., J. Lieberman, L. Gaidulis, and C. Ewing. 1976. Molecular abnormality of human $\alpha$-1-antitrypsin variant (PiZ) associated with plasma activity deficiency. *Proc. Natl. Acad. Sci. USA.* 73:1324–1328.

22. Owen, M. C., and R. W. Carrell. 1976. $\alpha_1$-antitrypsin:sequence of the Z variant tryptic peptide. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 79:247–249.

23. Kidd, V. J., R. B. Wallace, K. Itakura, and S. L. C. Woo. 1983. $\alpha$-1-Antitrypsin deficiency detection by direct analysis of the mutation of the gene. *Nature (Lond.).* 304:230–234.

24. Nukiwa, T., K. Satoh, M. L. Brantly, F. Ogushi, G. A. Fells, M. Courtney, and R. G. Crystal. 1986. Identification of a second mutation in the protein-coding sequence of the Z-type alpha-1-antitrypsin gene. *J. Biol. Chem.* 34:15989–15994.

25. Loebermann, H., R. Tokuoka, J. Deisenhofer, and R. Huber. 1984. Human $\alpha$-1-proteinase inhibitor. Crystal structure analysis of two crystal modifications: molecular model and preliminary analysis of the implications for function. *J. Mol. Biol.* 177:531–556.

26. Kelly, P. M., and M. J. Schlesinger. 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell.* 15:1277–1286.

27. Ananthan, J., A. L. Goldberg, and R. Voellmy. 1978. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock gene expression in chicken embryo fibroblasts. *Cell.* 15:1277–1286.

28. Kozutsumi, K., M. Segal, K. Normington, M.-J. Gething, and J. Sambrook. 1988. The presence of misfolded proteins in the endoplasmic reticulum signals the induction of glucose regulated proteins. *Nature (Lond.).* 332:462–464.

29. Kelly, P. M., and M. J. Schlesinger. 1982. Antibodies to two major chicken heat shock proteins cross react with similar proteins in widely divergent species. *Mol. Cell. Biol.* 2:267–274.

30. Schwartz, A. L., A. Ciechanover, R. Brandt, and H. J. Geuze. 1988. Immunoelectron microscopic localization of ubiquitin in hepatoma cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2961–2966.

31. Barbey-Morel, C., J. A. Pierce, E. J. Campbell, and D. H. Perlmutter. 1987. Lipopolysaccharide modulates the expression of $\alpha_1$-proteinase inhibitor and other serine proteinase inhibitors in human monocytes and macrophages. *J. Exp. Med.* 166:1041–1054.

32. Perlmutter, D. H., F. S. Cole, G. Goldberger, and H. R. Colten. 1984. Distinct primary translation products from human liver mRNA gives rise to secreted and cell-associated forms of complement protein C2. *J. Biol. Chem.* 259:10380–10385.

33. Roberts, B. E., and B. M. Paterson. 1973. Effect of translation of tobacco mosaic virus RNA and rabbit globin 6S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. USA.* 70:2330–2334.

34. Laemmli, U. K. 1970. Clevage of structural proteins during the assembly of the head of bacteriophage $T_4$. *Nature (Lond.).* 227:690–695.

35. Chirgwin, T. M., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5301.

36. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5205.

37. Bond, U., and M. J. Schlesinger. 1986. The chicken ubiquitin gene contains a heat shock promoter and expresses an unstable mRNA in heat-shocked cells. *Mol. Cell. Biol.* 6:4602–4610.

38. Caizzi, R., C. Caggese, and F. Ritossa. 1982. Modification of 70-kD heat shock protein of *Drosophila melanogaster*. *In* Heat Shock from Bacteria to Man. M. J. Schlesinger, M. Ashburner, and A. Tissieres, editors. Cold Spring Harbor Laboratory Press, New York. 251–257.

39. Strunk, R. C., A. S. Whitehead, and F. S. Cole. 1985. Pretranslational regulation of the synthesis of the third component of complement in human mononuclear phagocytes by the lipid A portion of lipopolysaccharide. *J. Clin. Invest.* 76:985–990.

40. Perlmutter, D. H., F. S. Cole, P. Kilbridge, T. H. Rossing, and H. R. Colten. 1985. Expression of the $\alpha_1$-proteinase inhibitor gene in human monocytes and macrophages. *Proc. Natl. Acad. Sci. USA.* 82:795–799.

41. Subjeck, J. R., and T. Shyy. 1986. Stress proteins systems of mammalian cells. *Am. J. Physiol.* 250:C1–C17.

42. Chirico, W. J., M. B. Waters, and G. Blobel. 1988. 70k heat shock-related proteins stimulate protein translocation into microsomes. *Nature (Lond.).* 332:805–810.

43. Deshaies, R. J., E. D. Koch, M. Werner-Washburne, E. E. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.).* 332:800–805.

44. Munro, S., and H. R. B. Pelham. 1986. An HSP-70-like protein in ER: identity with the 78-kD glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell.* 46:291–300.

45. Hendershot, L., D. Bole, and J. F. Kearney. 1987. The role of the immunoglobulin heavy chain binding protein in immunoglobulin transport. *Immunol. Today.* 8:111–114.

46. Hershko, A. 1988. Selectivity of ubiquitin-mediated protein breakdown: current status of our understanding. *In* Ubiquitin. M. Rechsteiner, editor. Plenum Publishing Corp., New York. 325–332.

47. Perlmutter, D. H., J. Travis, and P. I. Punsal. 1988. Elastase regulates the synthesis of its inhibitor, $\alpha$1-proteinase inhibitor, and exaggerates the defect in homozygous PiZZ $\alpha$1-proteinase inhibitor deficiency. *J. Clin. Invest.* 81:1774–1780.

48. Bathurst, I. C., J. Stenflo, D. M. Errington, and R. W. Carrell. 1983. Translation and processing of normal (PiMM) and abnormal (PiZZ) human $\alpha_1$-antitrypsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 153:270–274.