Endoplasmic Reticulum Stress Associated with Extracellular Aggregates

**EVIDENCE FROM TRANSTHYRETIN DEPOSITION IN FAMILIAL AMYLOID POLYNEUROPATHY**

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The hallmark of familial amyloid polyneuropathy (FAP) is the presence of extracellular deposits of transthyretin (TTR) aggregates and amyloid fibers in several tissues, particularly in the peripheral nervous system. The molecular pathways to neurodegeneration in FAP still remain elusive; activation of nuclear factor κB, pro-inflammatory cytokines, oxidative stress, and pro-apoptotic caspase-3 has been demonstrated *in vivo* in clinical samples and in cell culture systems. In this study, we investigated the involvement of endoplasmic reticulum (ER) stress response in FAP by showing activation of the classical unfolded protein response pathways in tissues not specialized in TTR synthesis but presenting extracellular TTR aggregate and fibril deposition. We also proved cytotoxicity by Ca$^{2+}$ efflux from the ER in cell cultures incubated with TTR oligomers. Taken together, these studies evidence ER stress associated with an extracellular signal in a misfolding disorder.

The hallmark of familial amyloid polyneuropathy (FAP) is the extracellular deposition of TTR in several tissues, particularly in the peripheral nervous system. The most common FAP-related point mutation in TTR is the substitution of a valine for a methionine at position 30 of the polypeptide chain. It was previously described that non-fibrillar TTR aggregates were present in sciatic nerves of asymptomatic individuals (2). It was previously described that non-fibrillar TTR aggregates were present in sciatic nerves of asymptomatic individuals, which also presented increased caspase-3 activation (3). Other stress-related molecules were also found to be linked to TTR aggregate-induced degeneration, namely pro-inflammatory cytokines and the nuclear factor κB (NF-κB) (4, 5).

Cellular stress conditions including altered redox status, increased protein synthesis, expression of misfolded proteins, and perturbation of calcium homeostasis can induce endoplasmic reticulum (ER)-specific stress response (6). This adaptive response is known as unfolded protein response and includes three basic signal transduction pathways mediated by the inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and double-stranded RNA-activated protein kinase-like ER kinase (PERK). All the main transducers are activated in response to accumulation of unfolded proteins, in a process dependent on the ER-resident chaperone BiP. BiP is a member of the heat-shock protein 70 family that transiently binds to newly synthesized, misfolded, or unassembled proteins in the ER lumen (7). The more effective chemical inducers of BiP expression are tunicamycin (which blocks N-glycosylation), thapsigargin (inhibitor of the ER Ca$^{2+}$ ATPase), and calcium ionophores, all affecting the ER folding capacity.

It is now widely accepted that the specific induction of BiP is indicative of ER stress due to its key role in the regulation of ER stress signaling. In non-stressed conditions, BiP binds to the luminal domains of IRE1, PERK, and ATF6, keeping them in the ER. Upon ER stress, BiP is released from IRE1 and PERK, permitting their oligomerization, phosphorylation, and activation (8–10), and ATF6 is transported to the Golgi, where it is cleaved to generate the p50 active form, which migrates to the nucleus and initiates transcription (11). On other hand, active PERK phosphorylates the eukaryotic initiation factor 2 α (eIF2α), resulting in attenuation of global mRNA translation (12). Fluctuations in the calcium pool are involved in cellular signaling upon ER stress (13). In the present study, we have investigated the expression of ER stress-related proteins (BiP, ATF6, and eIF2α) in tissues containing TTR deposits but not specialized in TTR synthesis and the contribution of calcium pools in this process.

**EXPERIMENTAL PROCEDURES**

**Subjects**—Human salivary gland (SG) biopsies were used in Western blots and immunohistochemical analyses as described previously (5). For the immunohistochemical studies, four sections each of TTR-V30M-associated FAP patients, asymptomatic carriers (FAP0), and control individuals were analyzed. For Western blots, a pool of SG from control and FAP0 individuals was applied.

**Animals**—Knock-out mice for heat-shock factor 1 and mouse endogenous TTR expressing human V30MTTR, char-
characterized previously (14), were used. The animals were housed in a controlled temperature room that was maintained under a 12-h light/dark period. Water and food were available *ad libitum*. All animal experiments were carried out in accordance with the European Community guidelines on the ethical use of animals and minimizing the number of animals used and their suffering. Experiments were carried out in 6-month-old mice (*n* = 10).

**Tissue Analyses—**Immunohistochemistry (IHC) on mouse dorsal root ganglia (DRG) and human SG sections was performed as described previously (5). Primary antibodies were: polyclonal goat anti-BiP (Santa Cruz Biotechnology, 1:50), polyclonal rabbit anti-TTR (Dako, 1:1,000), and polyclonal rabbit anti-eIF2α and ATF6 (Santa Cruz Biotechnology, 1:500). For fluorescence microscopy, antigens were visualized using donkey anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 568 (Molecular Probes). Semi-quantitative IHC analysis was performed using the Scion Image software (Scion Corp.). Results are presented as percentage of occupied area ± standard deviation (S.D.). Statistical significance was analyzed by the two-tailed Student’s *t* test. Congo red staining was performed as described previously (15).

**Proteins—**Recombinant wild type TTR was produced as described previously (16). Generation of oligomers was achieved after dialysis of TTR (at 250 μg/ml) against water, pH 7–8, and stirring for 7 days at room temperature. The preparations were positive by thioflavin-T spectrofluorometric assays. These conditions were chosen as a maximum yield is obtained in enzyme-linked immunosorbent assay tests using an antibody for oligomeric species (17).

**Cell Culture Assays—**ND7/23 cell line (mouse neuroblastoma × rat neuron hybrid) and SHSY5Y cell line. (Human neuroblastomas were from the European Collection of Cell Cultures.) Cells were propagated in 75-cm² flasks in monolayer and maintained at 37 °C in a humidified atmosphere of 95 and 5% CO₂. ND7/23 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin until 50–60% confluence. SHSY5Y cells were grown in medium containing Ham’s F12 (Invitrogen); minimum essential medium (Invitrogen) in a proportion of 1:1 supplemented with 2 mM glutamine, 1% non-essential amino acids (Sigma), 15% fetal bovine serum, and 100 units/ml of penicillin/streptomycin until 70–80% confluence. Subsequently, cells were washed twice with phosphate-buffered saline and treated for 16–24 h with 1 μM soluble TTR or 1 μM TTR oligomers in the presence or absence of different compounds as indicated; these included 2.5 μM thapsigargin (Sigma), 20 μM dantrolene (Sigma), or 1 μM xestospongin C (Calbiochem) in growing media containing 2% fetal bovine serum. Following treatment, cells were washed twice in ice-cold phosphate-buffered saline, trypsinized, and lysed in 50 μl of lysis buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100 and 1× protease inhibitors mixture (Amersham Biosciences)) by three cycles of freeze-thawing for Western blot analyses. Total protein concentration was determined using the Bio-Rad assay kit (Bio-Rad). For immunocytochemistry analysis, cells were grown in LAB-TEK (Nunc). After treatment, the cells were fixed with 2% paraformaldehyde, washed with phosphate-buffered saline, and incubated in blocking buffer (4% fetal bovine serum; 1% bovine serum albumin) for 30 min at 37°C. The primary antibody, eIF2α[P] (1:500, BIOSOURCE), was incubated at 4°C overnight. After washing twice with phosphate-buffered saline for 15 min, the corresponding secondary horse-radish peroxidase antibody was incubated for 30 min at room temperature. The reaction was developed with 3-amino-9-ethyl carbazole (Sigma) counterstained with hematoxylin and mounted with aqueous mounting medium.

**Caspase-3 Assay—**ND7/23 cells were propagated and treated as described above. Activation of caspase-3 was measured using the fluorometric caspase-3 assay kit (Sigma) following the manufacturer’s instructions. The remaining cell lysate was used to measure total cellular protein concentration with the Bio-Rad protein assay kit, using bovine serum albumin as standard.

**Western Blot—**Equal amounts of protein from treated cells or SG lysates were separated in 12% SDS-PAGE and transferred onto a nitrocellulose Hybond-C membrane (Amersham Biosciences) using a semidyry system. The primary antibodies used were goat polyclonal anti-BiP (Santa Cruz Biotechnology, 1:1,000), goat polyclonal anti-ATF6 (Santa Cruz Biotechnology, 1:200), polyclonal rabbit anti-eIF2α[P] (BIOSOURCE, 1:500), and mouse monoclonal anti-β-actin (Sigma, 1:5,000) for normalization. The enhanced chemiluminescence method (Santa Cruz Biotechnology) was used to develop the blots. Quantitative analysis of Western blot images was performed using the Scion software (Scion Corp.). Results are the average of three independent experiments and are presented as normalized density ± S.D.

**RESULTS**

**Increase in BiP Levels Occurs Only in DRG with TTR Deposition**—We analyzed the expression of the ER-resident chaperone BiP in DRG of 6-month-old mice. BiP was only detected in DRG with TTR deposits (Fig. 1i, panels A and B), whereas DRG negative for TTR extracellular deposits were negative for BiP (Fig. 1i, panels D and E). The link between BiP expression and TTR deposits was further analyzed by immunofluorescence. BiP and TTR did not co-localize; in fact, ganglia in close contact with TTR deposits (Fig. 1i, panel C, arrows) exhibit BiP staining by opposition with TTR-free ganglia (Fig. 1i, panel F). Increase in BiP staining was also observed in other organs that present extensive TTR deposition, namely the stomach (results not shown).

**Increase in BiP Levels Occurs in Human Salivary Glands with TTR Deposition**—The use of human salivary gland biopsies as a tool for diagnosis and research on FAP has been recently described (5). We investigated the expression of BiP by Western blot and IHC in normal and FAP SGs. Asymptomatic carriers of TTR V30M with extracellular TTR aggregate deposition clearly displayed increased BiP levels in comparison with normal control subjects (Fig. 1ii, panel D, Western blots). The Western blot results were further confirmed using IHC; as
compared with control biopsies, increase in BiP levels was clearly detected in asymptomatic carriers and in FAP patients (Fig. 1ii, panels A–C and quantification chart).

Small TTR Oligomers Induce BiP Expression Increase in a Cell Culture System, in a Process Dependent on Ca\textsuperscript{2+} Release from the ER—We used a mouse neuronal ND7 cell line to investigate the effect of small TTR oligomers on BiP expression. As shown in Fig. 2i, TTR oligomers induced an increase in the amount of BiP in a similar way as thapsigargin, a classical chemical inducer of BiP. As many of the cellular responses subsequent to ER stress are dependent on the action of the secondary messenger Ca\textsuperscript{2+}, we investigated whether the ER stress response triggered by TTR oligomers was mediated by the mobilization of Ca\textsuperscript{2+} from the ER to the cytosol using the specific inhibitors dantrolene (inhibitor of RyR channels) and xestospongin C (inhibitor of d-myo-inositol 1,4,5-trisphosphate-
shown in Fig. 3ii, TTR oligomers activated eIF2α as observed for BiP, further confirming the effect of TTR oligomers in ER stress.

Small TTR Oligomers Induce Caspase-3 Activation in a Cell Culture System, in a Process Dependent on Ca²⁺ Release from the ER—Finally, we tested whether the ER stress response triggered by TTR oligomers with subsequent mobilization of Ca²⁺ from the ER to the cytosol leads to cell death. We measured caspase-3 activation in the presence of specific Ca²⁺ inhibitors, dantrolene and xestospongin C, respectively. As depicted in Fig. 4, caspase-3 activation was only observed in cells treated with TTR oligomers; treatment with the inhibitors abrogated this cellular response.

**DISCUSSION**

This study clearly demonstrates the connection between the ER stress response and FAP. Increased levels of the ER-resident chaperone BiP were found, in a consistent way, in human biopsies of FAP patients, in TTR transgenic mouse DRG and cell line models. In mouse DRG, BiP expression is only visible in cells of the ganglia in close contact with TTR extracellular deposits, suggesting a cause-and-effect relationship. These results were further confirmed in human salivary gland biopsies, by Western blot and IHC. The increase in BiP level was visible in areas where mutant TTR is deposited (5).

BiP is a protein with a central role in the cellular response to stress, being a “sensor” of ER stress (7). In this way, it was possible to use BiP to assess the occurrence of ER stress in FAP; thus, we confirmed ER stress induction in FAP tissues by proteolysis of ATF6 and phosphorylation of eIF2α. Recent data reported that activation of NF-κB dependent on the phosphorylation of eIF2α as a substrate of serine for alanine at position 51 of the polypeptide chain resulted in inability to induce NF-κB activation (18). It is now well understood that the neurodegeneration pathways in FAP involve activation of NF-κB (5, 19), and it is possible that increase in eIF2α phosphorylation can partially account for NF-κB activation in TTR-related amyloidosis. Increase in BiP levels observed in our study on FAP tissues and transgenic mice possibly occurs also in other systemic amyloidoses.

Using an ND7/23 cell line, one of the few cell lines of peripheral neurons, and a human neuroblastoma cell line, we found that extracellular TTR oligomers can induce BiP expression and activation of eIF2α in a way similar to the chemical inducer thapsigargin. This increase in BiP levels involves the mobilization of two proteins involved in the unfolded protein response: ATF6 and eIF2α. In Western blots of SG from control individuals, the ATF6-p90 subunit did not vary between the two samples (Fig. 3i, panel D). An increase in eIF2α[P] in tissues from carriers and FAP patients versus controls was detected by Western blot (Fig. 3i, panel D) and by IHC in SG sections (Fig. 3i, panels A–C and quantification chart).

We next investigated whether eIF2α was activated by TTR oligomers in cell culture (using a human neuroblastoma cell line). As
tion of the secondary messenger Ca\(^{2+}\) from the ER as it can be blocked by the Ca\(^{2+}\) -channel inhibitors. The fact that xestospongin C suppressed the increase in BiP levels suggests that another secondary messenger, \(\alpha\)-myo-inositol 1,4,5-trisphosphate, can be involved in the process. Considering the fact that \(\alpha\)-myo-inositol 1,4,5-trisphosphate is generated in the plasma...
membrane by hydrolysis of phosphatidyl inositol 4,5-bisphosphate, one can speculate that an interaction of TTR oligomers with the membrane is an essential step in this mechanism.

We have previously shown (4) that incubation of cells (Schwannoma cell line) with TTR aggregates leads to caspase-3 activation and DNA fragmentation. At the time, the pathway/s underlying this finding were unknown; for the present work, we tested in additional experiments depicted in Fig. 4 whether dantrolene and xestospongin C, inhibitors of the mobilization of Ca\textsuperscript{2+} from the ER to the cytosol, could inhibit caspase-3 activation; indeed, this was the case, re-enforcing the notion of ER stress by extracellular TTR deposition as a toxic event in this system.

TTR synthesis occurs mainly in the liver and the choroid plexuses of brain (18), tissues devoid of TTR deposition in FAP. Instead, extracellular systemic deposition affects several organs and tissues with special involvement of the peripheral nervous system. Besides peripheral nerve, deposition in DRG contributes to the polyneuropathy characteristic of the disease. Early non-fibrillar aggregates occur extracellularly in presymptomatic stages of FAP (3, 20). The availability of a transgenic model with extracellular TTR aggregates in DRG was a unique tool to study molecular mechanisms underlying pathogenesis. In this regard, the clear BiP overexpression in DRG cells surrounded by TTR aggregates suggests indeed a role for ER stress associated with extracellular deposition and will be used to further dissect molecular mechanisms associated with neurodegeneration in FAP.

An increase in BiP levels was reported to be involved in the pathogenesis of inclusion body myositis, as assessed in both human biopsy samples and cultured muscle fibers (21). In contrast, BiP levels were not significantly different in the brains of patients with sporadic Alzheimer disease, or presenilin 1-mediated familial early onset Alzheimer disease as compared with control individuals (22) as well as in motor neurons in a transgenic mouse model of amyotrophic lateral sclerosis (23). This observation might relate to differences in aggregate localization in tissues and/or cell-specific susceptibility to aggregates. Future studies need to address novel intracellular signaling mechanisms related to extracellular stimuli such as protein aggregates in systemic amyloidoses to establish targeted therapeutic strategies. In a broader perspective, the finding of induction of ER stress by an extracellular protein can have implications in the way we understand signal transduction in general.

FIGURE 4. Activation of caspase-3 in ND7/23 cells incubated for 24 h with soluble and TTR oligomers in the presence and absence of dantrolene and xestospongin C. * p < 0.0002. Error bars indicate S.D.

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