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Protective effect of paraoxonase-2 against ER stress-induced apoptosis is lost upon disturbance of calcium-homeostasis

Sven Horke*, Ines Witte, Petra Wilgenbus, Sebastian Altenhöfer, Maximilian Krüger, Huige Li, Ulrich Förstermann

From the Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany.

*Address correspondence to:
Sven Horke, Ph.D.
Department of Pharmacology
Johannes Gutenberg University
Obere Zahlbacher Strasse 67
55131 Mainz
Germany
Email: horke@uni-mainz.de
Phone: +49 6131 39 33 192
Fax: +49 6131 39 36 611

Running head: Regulation of PON2 expression during ER stress and Ca^{2+}-disturbance

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Abstract

Paraoxonase-2 (PON2) is a ubiquitously expressed anti-oxidative protein, which is largely found in the endoplasmic reticulum (ER). Addressing the cytoprotective functions of PON2, we observed that PON2 overexpression provided significant resistance to ER stress-induced caspase-3 activation when the ER stress was induced by interference with protein modification (by tunicamycin or dithiotreitol), but not when ER stress was induced by disturbance of Ca^{2+} homeostasis (by thapsigargin or A23187). When analyzing the underlying molecular events, we found an activation of the PON2 promoter in response to all tested ER stress-inducing stimuli. However, only tunicamycin and dithiotreitol resulted in increased PON2 mRNA and protein levels. In contrast, when ER stress was caused by thapsigargin or A23187, we observed a Ca^{2+}-dependent active degradation of PON2 mRNA, elicited by its 5'untranslated region. In addition, thapsigargin and A23187 also induced PON2 protein degradation by a Ca^{2+}-dependent calpain-mediated mechanism. Thus, we provide evidence that independent mechanisms mediate the degradation of PON2 mRNA and protein after disturbance of Ca^{2+} homeostasis. Further, because Ca^{2+}-disturbance induces ER stress, but abrogates the otherwise protective function of PON2 against ER stress-induced apoptosis, we propose that the underlying cause of ER stress determines the efficacy of putative cellular defence mechanisms.

Key words:
gene regulation / calcium / paraoxonase / apoptosis / calpain / endoplasmic reticulum

Abbreviations used:
(s)XBP1, (spliced) X-box binding protein 1; 3-MA, autophagy-inhibitor 3-methyladenine; ALLN, calpain inhibitor-I N-acetyl-Leu-Leu-norleucinal; DTT, dithiotreitol; ER, endoplasmic reticulum; GRP78, glucose regulated protein of 78kDa; PON2, paraoxonase-2; ROS, reactive oxygen species; UPR, unfolded protein response pathway; UTR, untranslated region.

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Introduction

Upon synthesis, all proteins destined for transmembrane insertion or secretion translocate to the endoplasmic reticulum (ER). The ER is the site of protein amino-glycosylation, disulfide bond formation and proper folding of nascent proteins. In order to assist these processes, cells express molecular chaperones such as calnexin, glucose-regulated protein of 78 kDa (GRP78; BiP) and protein disulfide isomerase (PDI). Whereas formation and isomerisation of disulfide bonds is catalyzed by PDI [1], calnexin governs proper protein structure by retaining incorrectly folded proteins in the ER [2]. Ample evidence indicates that various stimuli, e.g. deprivation of amino acids, disturbance of glucose-/ Ca^{2+}-homeostasis or redox potential may interfere with protein maturation, which induces ER stress via the accumulation of unfolded proteins. This activates a conserved intrinsic stress response known as the unfolded protein response pathway (UPR). The UPR consists of three different orchestrated branches initiated by activation of ER stress transducers inositol-requiring protein 1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF6); these enable cellular responses ranging from stress relieve to ER remodelling, protein degradation, autophagy and apoptosis [3]. A key mediator of the UPR is GRP78. Although still under debate, one model of how ER stress is sensed suggests that under unstressed conditions, GRP78 binds to IRE1, PERK and ATF6 to keep them in an inactive state. Upon stress, however, the ER chaperone GRP78 is attracted by and bound to unfolded proteins, which results in a loss of interaction of GRP78 with IRE1, PERK and ATF6 [4, 5]. This activates these integral membrane proteins that thereby sense the folding status in the ER lumen and link this information to cytosolic events. As such, IRE1 performs unusual splicing of X-box-binding-protein-1 (XBP1) mRNA, PERK phosphorylates eIF2a protein and ATF6 is proteolytically processed in order to release a soluble cytosolic portion that acts as transcription factor [6]. As a consequence, general translation is diminished and gene expression is rapidly altered in order to alleviate stress or undergo apoptosis.

In vivo relevance of the UPR is highlighted by the fact that this pathway is critically involved in several diseases [7], such as Alzheimer [8], apoptosis of pancreatic β-cells [9], diabetes mellitus and atherosclerosis [10, 11]. The latter is characterized by an enhanced production of reactive oxygen species (ROS) and inflammatory responses of vascular cells and macrophages, both of which contributes to the pathophysiology of atherosclerosis [12-14]. For vascular endothelial cells it is known that oxidized low density lipoprotein particles (oxLDLs) or bioactive components thereof can induce oxidative stress [15], ER stress and inflammatory cytokine production [16]. Similarly, it was shown that accumulation of free cholesterol, which may represent a likely cause of cell death in later stages of atherosclerotic lesions, causes macrophage apoptosis via ER stress [17]. Thus, an understanding of underlying molecular mechanisms is mandatory, as it determines UPR induced gene expression, regulation of molecular stabilities as well as pro- and anti-apoptotic factors.

We recently described that another cellular enzyme, paraoxonase-2 (PON2) is involved in ER stress [18]. The paraoxonase family of proteins consists of three highly similar enzymes, PON1, PON2 and PON3 [19] with characterized enzymatic activities [20, 21]. Although it is known that PON2 plays a dominant role in the inactivation of certain lactone(s) involved in quorum sensing of *Pseudomonas aeruginosa* [22, 23], natural endogenous substrates remain relatively elusive. In the case of PON2, we reported the existence of two distinct, glycosylated splice-variants [18]. In vivo studies using transgenic and / or deficient mice revealed anti-atherogenic properties of all three PONs [24-27]. In accordance, we and others reported that PON2 significantly reduced intracellular oxidative stress [18, 28]. Although regulation of PON2 expression remained largely enigmatic, it is known that PON2 expression is induced by both ER stress [18] and oxidative stress [29] and appears to be regulated, at least in part, by PPARγ and AP1 pathway activation [30]. Prompted by the observation that PON2 overexpression rendered endothelial cells significantly more tolerant to ER stress-induced apoptosis (i.e. caspase-3 activation), which was opposed after down-regulation of PON2 [18], we asked as to whether PON2 was generally protective against ER stress-induced apoptosis. We here report that the protective and anti-apoptotic effect of PON2 was not a general phenomenon; it was absent, when the ER stress was induced by Ca^{2+}-deregulation. The current study was designed to uncover the underlying molecular events leading to a loss of protection by PON2 under specific ER stress conditions.

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Experimental

Cultivation of EA.hy 926 cells and UPR induction

Human endothelial EA.hy 926 cells were cultured in Dulbeccos modified eagle medium (without phenol red; Sigma) containing Na-pyruvate (PAA), antibiotics penicillin / streptomycin, HAT supplement, Glutamax (Invitrogen) and 10% fetal calf serum (Perbio). Cells were cultured at 37°C in a humidified atmosphere at 10% CO_2. Medium for cultivation of EA.hy 926 cells stably overexpressing PON2-isoform1-green-fluorescent-protein (PON2-iso1-GFP) or PON2-isoform1-hemagglutinin (PON2-iso1-HA) [18] contained geneticin (300 µg/ml; PAA) in addition. Cells were confluently seeded in 96-well plates (2.0 x 10^4/well) or 24-well plates (1 x 10^5/well). The next day, DTT (solved in water), tunicamycin (Sigma), thapsigargin (Invitrogen), A23187 (Sigma) or solvent control dimethylsulfoxid (DMSO) was added to fresh culture medium. Maximum DMSO concentration did not exceed 0.5%. Efficient UPR induction was verified in every experiment by confirming enhanced expression of GRP78 mRNA and / or protein.

Inhibitors, cell lysis and Western blotting

Cells were seeded and treated as outlined above. Unless otherwise indicated, the following inhibitors (or solvent control) were supplemented for 1 h in complete growth medium before addition of UPR inducing compounds: proteasome-inhibitors lactacystin (10 – 50 µM; Cayman Chemicals) or MG132 (10 µM); autophagy-inhibitor 3-methyladenine (3-MA; 1 – 5 µM); calpain inhibitor-I N-acetyl-Leu-Leu-norleucinal (ALLN; 1 – 5 µM); cycloheximide (5 µg/ml); actinomycin D (5 µg/ml)(all from Sigma); BAPTA/AM (10µM; Calbiochem). Inhibitors remained present throughout subsequent addition of UPR inducing compounds. At the indicated time points, medium was aspirated, cells were washed with phosphate-buffered saline and lysed by addition of ice-cold lysis buffer (100 mM Tris/HCl pH 7.4, 200 mM NaCl, 2% Triton X-100, both protease and phosphatase inhibitor cocktails Complete and PhosphoStop (Roche)), followed by 20 min rocking at 4°C and mechanical disruption. Finally, lysates were cleared by centrifugation (30 min, 12,000 x g, 4°C). Protein content was determined using the BCA protein assay reagent (Pierce). Western blotting was performed as described earlier [18]. The following primary antibodies (all anti human) were used: mouse monoclonal-anti-α-tubulin (Ab-2 DM1a; Dianova), goat anti-GRP78 (C-20; Santa Cruz) and rabbit-anti-PON2 [18].

Cloning of the PON2 5'untranslated region (UTR)

Earlier we described the generation of a pGL4.10 plasmid (Promega) containing genomic DNA of the PON2 gene ranging from 2249bp upstream of the 5'end to position +112 [18]. This plasmid was digested with restriction enzyme Nco-I, which cuts out a 147bp fragment (containing parts of Exon-1, Intron-1/2 and some vector backbone); religation of the larger fragment (6444bps) results in a plasmid harbouring the PON2 promoter fragment (-2249 to -1), directly followed by the putative PON2 5'UTR and the firefly luciferase gene. Thus, the putative PON2 5'UTR was cloned based on the Genatlas / Gene database; its sequence consists of 32bps 5'-GGAGCGAGGCAGCGCGCCCGGCTCCCGCGCC-3'. Because this sequence was cloned according to public database entries, it may not represent the complete PON2 5'UTR.

Cloning of the PON2 3'UTR by rapid amplification of cDNA ends (3'RACE)

Using EA.hy 926 total RNA, PON2 mRNA 3'UTR was amplified by 3'RACE with primers GAGAGATCTAGACTCGTCAGAGGTTCTCCGCATCCAG and CATCCTGGTCGAGCTGGGGCCGGCCTTTTTTTTTTTTTTTTTTTTTTG with the Superscript-II RT-PCR system (Invitrogen) according to the supplier's instructions. Amplified DNA was gel-purified, blunt ends generated using Klenow-fragment, the DNA inserted into zero-blunt pCR4-TOPO plasmid (Invitrogen) and sequenced. The amplified sequence contains the 3'UTR and a limited base pair number of the coding sequence. From this plasmid, the 3'UTR was amplified by PCR using the same reverse primer as before together with the sense-primer GACAGACATCTAGAATTGTACTTTTGGCATGAAAGTGCG. The amplified 3'UTR was inserted into the plasmid pGL4-PON2-promoter [18] C-terminal to the luciferase gene by standard cloning procedures using restriction enzymes FseI and XbaI followed by seqencing.

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RNA extraction, quantitative real-time RT-PCR (qRT-PCR) and mRNA decay analysis
To analyze mRNA decay, cells were solvent-treated (control) or were pre-treated for 4h with the UPR inducing compound as indicated in the figure legends. Then, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB; 60 µM; Sigma) or actinomycin D (5 µg/ml) was added to inhibit RNA synthesis. UPR inducing compounds remained present throughout this treatment. At the indicated time points, total RNA was extracted from EA.hy 926 cells cultured in 24-well plates using E.Z.N.A. total RNA kit (Omega Bio-tek Inc) according to the suppliers instructions. Then, 80 ng RNA was used for cDNA generation using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the suppliers instructions. Finally, RNA expression levels were quantitatively analyzed using 2 µl cDNA, 1 U Taq-polymerase (PeqLab), 0.4 µl 10 mM dNTPs and 2 µl per primer per 20 µl reaction in a Bio-Rad iCycler (Bio-Rad). Pre-designed, validated Taqman primers for detection of PON2 or GRP78 were purchased from Applied Biosystems. Additionally, the following Taqman primers (synthesized by MWG) were used: for spliced XBP1 GGAGTTAAGACAGCGCTTG (sense); GCACCTGCTGCGGACTC (antisense); GAAGCCAAGGGGAATGAAGT (probe); for glycerine-aldehyde-3-phosphate-dehydrogenase (GAPDH) CAACAGCCTCAAGATCATCAGC (sense); TGGCATGGACTGTGGTCATGAG (antisense); CCTGGCCAAGGTCATCCATGACAAC (probe); for EGFP CTGACCCTGAAGTTCATCTGC (sense); AAGTCGTGCTGCTTCATGTG (antisense); TCGTGACCACCCTGACCTAC (probe); for firefly luciferase TGCAAAAGATCCTCAACGTG (sense); AATGGGAAGTCACGAAGGTG (antisense); AAGACCGACTACCAGGGCTT (probe); for renilla luciferase ATTGGTATGGGCAAATCAGG (sense); GGCCGACAAAAATGATCTTC (antisense); TCTTACTGCATGGTTTGAACTTCT (probe). qRT-PCR was performed as described earlier [18]. All mRNA levels were normalized to GAPDH mRNA (or renilla luciferase mRNA where indicated) from the very same samples and calculated by the $2^{[-\Delta\Delta C(T)]}$ method. When analyzing the function of PON2 mRNA UTRs, cells were co-transfected with a plasmid allowing for constitutive renilla luciferase expression and the indicated pGL4-PON2-promoter plasmids (including the 3'- or 5' UTR). After the specific treatment, RNA was prepared as above, but additional extensive DNase-I on-column digestion (Omega Bio-tek Inc.) was performed. Principally, this method is somewhat susceptible to unwanted amplification of remaining plasmid DNA in RNA preparations. Therefore, we controlled effective plasmid digestion by including RNA preparations without DNase-I digestion in every experiment. In subsequent qRT-PCR analyses, these differed by a factor of >20 (data not shown).

Software, statistics and image acquisition
Device specific software provided by the suppliers was used. In addition, GraphPad Prism software was used for any calculations of collected data, for non-linear regression curve fitting or statistical evaluation using 1-way ANOVA with Bonferronis multiple comparisons post-test (see figure legends). A probability level of 0.05 was considered significant. Adobe Photoshop software was used for image acquisition of scanned Western blots. If at all, solely brightness and / or contrast were changed and adjusted simultaneously for all lanes and areas of any blot.

All other relevant materials such as plasmids, promoter reporter analyses and caspase-3/7 activity determination have been described recently [18].

Results

PON2 protects against ER stress-induced apoptosis unless ER stress is caused by disturbance of Ca^{2+}-homeostasis
Prolonged ER stress can induce apoptosis, which can be monitored by the activity of executioner caspase-3. Here we determined the effect of PON2 overexpression on caspase-3/7 activation in response to a 16h-treatment with varying concentrations of different ER stress inducing compounds, namely tunicamycin, DTT or thapsigargin. These induce ER stress via distinct mechanisms: DTT obliterates protein structures, tunicamycin interferes with protein amino-glycosylation and thapsigargin disturbs cellular Ca^{2+} homeostasis.

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As control, we first determined the maximum caspase-3 activation by treating naïve EA.hy 926 cells with pro-apoptotic staurosporin (Figure 1A). Within the concentrations tested, 0.3 µM staurosporin resulted in the highest (approximately 5-fold) stimulation. We then treated EA.hy 926 cells with increasing concentrations of DTT and observed concentration-dependent caspase-3 activation (Figure 1B). In cells stably overexpressing PON2-iso1-GFP or PON2-iso1-HA, maximum DTT-induced caspase-3 activation was markedly reduced. Similar results were obtained with tunicamycin (Figure 1C). Here, PON2 overexpressing cells showed only very limited caspase-3 activation. However, different results were obtained for thapsigargin-induced caspase-3 activation. PON2 overexpression somewhat decreased the potency of thapsigargin to induce caspase-3 activation, but the maximum effect was not significantly reduced (Figure 1D).

Stimulators of ER stress enhance PON2 promoter activity

Addressing underlying molecular events causing loss of protection of PON2 against thapsigargin-, but not tunicamycin- or DTT-induced caspase-3/7 activation, we analyzed the relative alterations in PON2 gene expression caused by the tested compounds. We recently described the generation of a plasmid harbouring a firefly luciferase gene under the expressional control of a PON2 promoter fragment (pGL4-PON2-promoter; corresponding to region -2249 to -1), which was used for reporter gene analyses [18]. Within this promoter fragment, sequences with high homology to ER stress-responsive *cis*-acting elements can be found, such as ER stress element (ERSE)-like sequence [31] and the UPR element [32]. This plasmid was used to evaluate the effect of different UPR inducing compounds on PON2 promoter activation. Transiently transfected human endothelial EA.hy 926 cells were treated for 16h with varying concentrations of DTT, tunicamycin or thapsigargin. A concentration dependent increase in promoter activity was observed with all three substances (Figure 2). With the highest concentrations tested, a 5-fold (tunicamycin; Figure 2B) to 9-fold (DTT, thapsigargin; Figures 2A, C) enhancement was observed. Interestingly, there was no decline in promoter activity even with concentrations that evidently induced pro-apoptotic executioner caspase-3 (see Figure 1). Non-specific induction of the PON2 promoter by serum components was excluded by the similar induction levels observed in the absence or presence of serum (Figure 2D).

Tunicamycin and DTT induce whereas thapsigargin declines PON2 mRNA expression

We next determined the effect of the UPR inducers on endogenous PON2 mRNA levels. EA.hy 926 cells were treated with constant concentrations of DTT (2.5 mM) or tunicamycin (1 µg/ml) and samples were taken at different time points for PON2 mRNA quantification by real-time qRT-PCR. UPR induction was verified by significantly increased levels of endogenous GRP78 mRNA (not shown), whose induction is considered characteristic of ER stress. Figure 3A shows that DTT led to a moderate increase (~180%) of endogenous PON2 mRNA in a time-dependent fashion. Similarly, tunicamycin treatment caused enhanced PON2 mRNA expression (Figure 3B). Here, PON2 mRNA levels increased approximately 3-fold after six hours. Upon exposure to thapsigargin, however, the reverse result was obtained. Specifically, we observed both a time-dependent and concentration-dependent decline in PON2 mRNA levels after thapsigargin treatment (Figures 3C+D, respectively). Hence, although DTT, tunicamycin and thapsigargin equally induce both the UPR pathway and PON2 promoter, they show opposed effects on endogenous PON2 mRNA levels.

Principally, a thapsigargin-induced decrease in PON2 mRNA levels may result from both a discontinued transcription and an active degradation. The latter was tested by analyzing PON2 mRNA stability. To this end, cells were pre-treated with lowered, non-cytotoxic concentrations of DTT (2.5 mM), tunicamycin (0.5 µg/ml) or thapsigargin (1 nM) for 4 hours to induce the UPR followed by inhibition of RNA synthesis by addition of DRB. In parallel, basal PON2 mRNA decay in indigenous EA.hy 926 cells was monitored. These analyses revealed a surprisingly long half-life of PON2 mRNA. Even 24 hours after inhibition of RNA synthesis by DRB, on average 70% of PON2 mRNA remained present (Figure 4). Similar results were obtained using actinomycin D (see below). This is in agreement with our observation of a relatively long half-life (approx. 2-3 days) of PON2 protein, which was seen in experiments based on treatment with RNA interference (not shown) or cycloheximide (see below). UPR induction in EA.hy 926 cells either by DTT or tunicamycin (Figures 4A+B, respectively) did not significantly change decay of endogenous PON2 mRNA. UPR induction by thapsigargin, however, resulted in a significantly faster decay of PON2 mRNA (Figure 4C). Already at the first time point analyzed (4 hours), PON2 mRNA was degraded by approx. 25%,

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whereas no significant decay was observed at this time point with the other conditions tested. Thus, in contrast to DTT or tunicamycin, thapsigargin treatment of EA.hy 926 cells induced an active degradation of PON2 mRNA.

A disturbed Ca^{2+}-homeostasis, but not the secondary activation of the UPR pathway causes PON2 mRNA decay

Thapsigargin blocks the sarco-/endoplasmic reticulum calcium-ATPase SERCA, which depletes the ER calcium storage thereby causing ER stress. We next investigated whether the observed PON2 mRNA decay upon thapsigargin treatment results from the primary stimulus, namely a deregulated intracellular Ca^{2+}-signalling, or from a secondary thapsigargin-specific UPR induction. For this purpose, naïve EA.hy 926 cells were treated for 16h with thapsigargin (10 nM) or the potent cytosolic Ca^{2+}-chelating compound BAPTA/AM (10 µM), or combinations thereof. While BAPTA/AM treatment by itself resulted in somewhat reduced PON2 mRNA levels, it clearly inhibited the thapsigargin-induced degradation of PON2 mRNA (Figure 5A). The UPR pathway was still activated in the thapsigargin+BAPTA/AM treated samples as evidenced by more than 15-fold and 11-fold increases in GRP78 mRNA and spliced X-box binding protein-1 (sXBP1) mRNA, respectively (Figures 5B+C). This suggests that PON2 mRNA degradation results from deregulated Ca^{2+}-signalling rather than the UPR. Therefore we explored if a mechanistically different disturbance of intracellular Ca^{2+}-homeostasis results in similar effects. Indeed, EA.hy 926 cells treated with the calcium ionophore A23187 showed significantly reduced PON2 mRNA levels, which could be inhibited by BAPTA/AM (Figure 5D). Similar to thapsigargin, also A23187 produced a marked enhancement of GRP78 and sXBP1 mRNA levels, even in the presence of BAPTA/AM (more than 16-fold and 18-fold, respectively; Figures 5E+F).

Next we examined if the PON2 mRNA Ca^{2+}-responsive element is located in the untranslated region(s) (UTR) or e.g. the coding sequence. To this end, we performed 3'RACE experiments and cloned the 3'UTR of PON2, as well as the 32bps-long 5'UTR (according to public database entries) into the above mentioned pGL4-PON2-promoter plasmid. As a result, this plasmid was modified such that the encoded firefly luciferase mRNA was N- or C-terminally fused to the 5'UTR or 3'UTR of endogenous PON2 mRNA, respectively. Basal expression was comparable for all constructs and was independent from the addition of 5' or 3'UTRs (not shown). As shown in Figure 5G, thapsigargin treatment enhanced PON2 promoter activity and resulted in a marked increase of firefly luciferase mRNA (in agreement with firefly luciferase activity, Figure 2C). However, after PON2 5'UTR was fused to firefly luciferase mRNA, thapsigargin caused pronounced destabilization of this unrelated mRNA (Figure 5G). The 3'UTR of PON2 showed no such effect (Figure 5G). Also, the Ca^{2+}-independent stressor tunicamycin did not induce decay of the firefly luciferase mRNA independent of the addition of the PON2 5' or 3'UTR (Figure 5H).

Thus, we presumed that the Ca^{2+}-responsive element of endogenous PON2 mRNA is located in its 5'UTR. In agreement with this assumption, firefly luciferase mRNA levels in thapsigargin-treated cells overexpressing the 5'UTR construct were reduced to less than control levels (~80% in thapsigargin treated relative to untreated cells; Figure 5G). As further evidence for the occurrence of an Ca^{2+}-responsive element in the 5'UTR of PON2 mRNA we show that thapsigargin treatment did not cause degradation of overexpressed PON2-GFP mRNA (Figure 5J), which solely contains the PON2 coding sequence without UTRs.

DTT and tunicamycin enhance PON2 protein levels, whereas disturbed Ca^{2+}-homeostasis causes its degradation

To test if disturbed Ca^{2+}-homeostasis also results in PON2 protein degradation, similar experiments as above were performed and protein levels were controlled by Western blotting. As shown recently [18] and in Figure 6A, DTT (2.5 mM) treatment of EA.hy 926 cells resulted in a moderate, time-dependent increase in PON2 protein expression. UPR activation was verified by probing for GRP78 (not shown). Similarly, tunicamycin treatment caused enhanced PON2 protein expression in a concentration- and time-dependent manner (Figures 6B+C, respectively). Since both isoforms of PON2 are glycosylated – which is inhibited by tunicamycin – newly synthesized PON2 isoforms 1 + 2 appear with lower apparent molecular weights. *De novo* synthesis of these isoforms is indicated by the fact their emergence is obliterated by cycloheximide (Figure 6C). Importantly, no degradation of PON2 protein

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was observed even with concentrations of tunicamycin that strongly activate pro-apoptotic caspase-3 (Figure 1C).
By contrast, thapsigargin treatment resulted in a concentration-dependent degradation of PON2 protein (Figure 7A). This was partially inhibited by pre-treatment with BAPTA/AM (Figure 7B). Similar to the results obtained for PON2 mRNA, A23187 mimicked the thapsigargin-mediated effects on PON2 protein; A23187 treatment also caused PON2 degradation, which was mostly prevented by BAPTA/AM (Figures 7C+D). Finally, we investigated the underlying mechanism of this active PON2 degradation. Thapsigargin- or A23187-mediated increases in cytosolic Ca^{2+}-concentrations may give rise e.g. to proteasomal degradation of proteins, calpain protease activation or autophagy. In order to distinguish between these possibilities, EA.hy 926 cells were treated for 16h with solely 10 nM thapsigargin or together with inhibitors of proteasome activity (MG132, 10 μM, not shown) and lactacystin (50 μM), calpain inhibitor-I (N-acetyl-Leu-Leu-norleucinal, ALLN, 5 μM) or an inhibitor of autophagy (3-methyladenine, 3-MA, 5 μM). As shown in Figure 7E, thapsigargin-induced decay of PON2 did not result from proteasomal activity or autophagy, because the well-known and potent inhibitors lactacystin and 3-MA did not prevent PON2 degradation. By contrast, we show that PON2 protein is subject to Ca^{2+}-activated calpain protease activity, because the calpain inhibitor-I ALLN inhibited thapsigargin-induced PON2 decay. In summary, PON2 protein stability negatively correlates with increased cytosolic Ca^{2+}-concentrations and calpain activation. Similar to endogenous PON2, this also applies to stably overexpressed PON2-GFP, which is degraded upon thapsigargin exposure in a time-dependent manner (Figures 7F+G, respectively). Thus, the degradation of both endogenous and overexpressed PON2 in response to Ca^{2+}-disturbances elucidates the cause of lack of protection by PON2 overexpression against thapsigargin-induced apoptosis. To demonstrate the close correlation between thapsigargin-induced PON2 mRNA- and protein degradation on the one hand, and caspase-3 activation on the other hand, these parameters were plotted in one graph in Figure 7H.

Discussion

ER stress is critically involved in the pathogenesis of diseases such as diabetes [10] or neurodegenerative disorders [7]. In atherogenesis, enhanced uptake of free cholesterol by macrophages has been shown to induce ER stress [33]. Due to the central position of the ER and the UPR, there is significant interest in factors modulating the cellular pro- and anti-apoptotic response to ER stress. Since the underlying cause of ER stress appears critical for the cellular response, we investigated whether the recently discovered anti-apoptotic function of PON2 was applicable for several forms of ER stress or if it was dependent on the inducing stimulus and if different causes of the stress similarly affected PON2 gene expression.
Stable overexpression of PON2 decreased caspase-3 activation stimulated by DTT or tunicamycin, but not that stimulated by thapsigargin. Most probably, this is due to the fact that deregulation of cytosolic Ca^{2+}-levels in response to thapsigargin or A23187 caused a rapid degradation of both endogenous and even the overexpressed PON2. Because even strongly pro-apoptotic concentrations of tunicamycin did not result in PON2 degradation, this implies that tunicamycin-induced apoptosis does not elicit a secondary rise in cytosolic Ca^{2+}, at least not to the extent caused by yet low concentrations of thapsigargin or A23187. Alternatively, our data could suggest that the protective effect of PON2 lies upstream of caspase-3 and that PON2 does not e.g. interfere with caspase-3 activation directly. In addition, it appears unlikely that UPR-accompanying ROS production is the underlying common pro-apoptotic mechanism, as this would probably be mostly reduced by overexpression of anti-oxidative PON2. Moreover, it may suggest that PON2 has different effects on the pro-apoptotic pathways activated by the tested compounds. Thapsigargin mediates apoptosis by mitochondrial caspase-dependent death cascades, Ca^{2+}-dependent anti-apoptotic phosphatidylinositol 3-kinase / MAPK activation [34] and depends on enhanced nitric oxide synthase activity [35]. Tunicamycin-induced cell death, in contrast, is facilitated rather by ER specific apoptosis pathways, i.e. by ER-resident protein calnexin [36] or caspase-2 /-9 activation [37]. Due to its ER localization [18, 38], PON2 may primarily interfere with ER derived apoptotic pathways. In line with this, neither PON2 overexpression nor knock-down altered nitric oxide production in EA.hy 926 cells (M.K. & S.H.; unpublished observations), which is considered essential for thapsigargin-induced apoptosis [35].

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When addressing ER stress-induced alterations in PON2 gene expression, we found that a heterogeneous group of compounds whose common denominator is the induction of ER stress all stimulated PON2 promoter activity. Provided the PON2 promoter 2249-bp fragment contains all relevant regulatory sites, our data indicate that transcriptional activation of UPR-responsive genes is the consequence of a secondary UPR pathway activation and is independent of the UPR-inducing stimulus. ER stress responsive promoter elements such as ERSE, ERSE2, UPRE or others are not assigned to specific forms of stress (e.g. disturbed Ca^{2+}-homeostasis vs. hindered protein modification). PON2 induction thus differs from the UPR-mediated induction of upstream, ER stress-responsive transcriptional activators such as ATF6 and XBP1, which appears stimulus-specific [39]. Additionally, ER stress transducers such as IRE1, PERK, ATF6 and XBP1 are activated with different time kinetics in order to meet cellular requirements for protein refolding / degradation, or apoptosis [40, 41].

At the level of mRNA turn-over and / or translation, however, mechanism(s) evidently exist that can distinguish between DTT-, tunicamycin-, thapsigargin- or A23187-induced ER stress. These mechanism(s) may sense whether the increased expression of a specific protein is beneficial for stress relieve and may regulate the mRNA / protein stability accordingly. This is in line with a global profiling of ER stress-regulated mRNAs, where clear differences were found between thapsigargin or tunicamycin treatment of the same cells [42]. In the current study, all ER stress-inducing compounds tested resulted in a transcriptional activation of the PON2 gene. As expected, DTT and tunicamycin moderately increased PON2 mRNA and protein levels. In contrast, thapsigargin and A23187 produced a pronounced degradation of endogenous PON2 mRNA and protein. It is currently unclear which RNase activity (or other destabilizing mechanism) mediates the decay of PON2 mRNA. One candidate protein could be IRE1, which possesses RNase activity and was recently found to mediate the ER stress-induced degradation of specific mRNAs in *Drosophila* [43]. IRE1 activation in our experimental setup was evidenced by markedly increased levels of spliced XBP1 mRNA. However, because BAPTA/AM prevented PON2 mRNA degradation, but not UPR induction or IRE1 activation, it appears unlikely that IRE1 fulfils this function in our model. Rather, increased cytosolic Ca^{2+}-levels may activate mechanisms destabilizing PON2 mRNA. Ca^{2+} is known to regulate mRNA stability through stabilizing or destabilizing proteins such as HuR or AUF-1 [44]. Interestingly, these proteins mostly act on UTRs, which is in line of our finding that Ca^{2+}-induced PON2 mRNA decay depends on the 5'UTR. Alternatively, Ca^{2+}-stimulated RNase L [45] may represent a mechanism for PON2 mRNA decay. In contrast to PON2, it has recently been shown that ER stress rather than cytosolic Ca^{2+} upregulated HIF1-α mRNA [46]. In this context, it is to be noted that the PON2 mRNA 5'UTR mediated the degradation of an unrelated (firefly luciferase) mRNA upon cytosolic Ca^{2+}-increase; however, this effect was less pronounced when compared to results obtained for the Ca^{2+}-induced degradation of endogenous PON2 mRNA. Thus, one may speculate that additional sequences and / or structures found in the native PON2 mRNA may augment the destabilizing effect of the 5'UTR upon cytosolic Ca^{2+}-increase.

PON2 is a long-lived and stable protein (see Figure 6C). Nevertheless, thapsigargin (and also Ca^{2+}-ionophore A23187) stimulated an active, rapid and calpain-mediated degradation of this protein (Figure 7). This is in agreement with the finding that an increase in intracellular Ca^{2+} can be a potent activator of cytoplasmic proteases, thereby contributing to cell death [47]. To our knowledge, it is unknown whether (patho)-physiological settings exist, in which Ca^{2+}-stimulated down-regulation of PON2 is observed. Given that PON2 protects cells from apoptosis (at least under some ER stress conditions), its decay upon Ca^{2+}-disturbance may contribute to the deleterious effects associated with various pathophysiological situations. Examples could include ischemia, which perturbs Ca^{2+}-homeostasis and activates calpains [48], or the effects of oxysterols, which lead to increased cytosolic Ca^{2+}-levels and endothelial cell death [49]. Therefore, it is mandatory to unravel the anti-apoptotic mechanism of PON2 and to explore if this is a general phenomenon or restricted to the UPR.

We present several pieces of evidence indicating that two independent mechanisms mediate the degradation of PON2 mRNA and the destabilization of PON2 protein following disturbance of Ca^{2+} homeostasis: (1) PON2 protein per se is very stable; protein levels were unchanged 24h after actinomycin D or 32h after cycloheximide (Figures 7F and 6C, respectively). Given this protein stability, an accelerated decay of PON2 mRNA in response to thapsigargin or A23187 cannot explain the parallel decay of PON2 protein (down to 42% after 16 h, Figure 7F); (2) PON2-specific siRNA results in a 90% reduction of PON2 mRNA after 1 day, but PON2 protein is not reduced before 3 days

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(data not shown); (3) PON2 protein degradation occurs independent of the mRNA's 5'UTR, because PON2-GFP protein is degraded after Ca^{2+}-disturbance although the PON2-GFP mRNA lacks any UTR; (4) We show that calpain proteases mediate Ca^{2+}-stimulated PON2 protein decay.
Taken together, our findings suggest that UPR-induced activation of the PON2 promoter is a relatively non-specific effect independent of the inducing stimulus. In contrast, the subsequent regulation of PON2 mRNA and protein stability seems to be specific for the stress-inducing mechanism(s). Increases in cytosolic Ca^{2+}-concentrations lead to rapid degradation of both PON2 mRNA and protein by separate, unrelated mechanisms. We thus demonstrate that the mechanisms causing ER stress are of major importance for PON2 regulation and its protective function against ER stress-induced caspase-3 activation.

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Figure legends

Figure 1: PON2-mediated tolerance to ER stress-induced apoptosis is lost after disturbance of Ca^{2+}-homeostasis. (A) To test maximum caspase-3/7 activation, naïve EA.hy 926 cells were treated for 16h with the indicated concentrations of apoptosis-inducing compound staurosporin with subsequent caspase-3/7 activity determination. Alternatively, naïve or PON2-iso1-HA or PON2-iso1-GFP overexpressing EA.hy 926 cells were treated for 16h with various concentrations of DTT **(B)**, tunicamycin **(C)** or thapsigargin **(D)**. Then caspase 3/7 activity was determined. Symbols represent mean ± SEM; n=6-14. Caspase-3/7 activation in PON2-GFP / PON2-HA overexpressing cells was significantly lower than in naïve EA.hy 926 cells with concentrations equal to or higher than 10 mM DTT or 1 µg/ml tunicamycin ($P<0.05$; $P<0.001$, respectively).

Figure 2: UPR stimulates PON2 promoter activity independent from the nature of ER stress. EA.hy 926 cells were co-transfected with plasmids encoding for a firefly luciferase gene under the expressional control of the PON2 promoter fragment and with a plasmid for constitutive expression of renilla luciferase (normalization). Four hours after transfection, cells were treated for 16h with increasing concentrations of DTT **(A)**, tunicamycin **(B)** or thapsigargin **(C)**. Finally, firefly luciferase activity reflecting PON2 promoter induction was recorded, normalized to renilla luciferase activity and expressed as fold induction. **(D)** Addressing if serum components influence PON2 promoter activity, EA.hy 926 cells were seeded, transiently transfected with the PON2 promoter construct, serum deprived for 16h, and finally either left untreated or stimulated by adding medium with 10% fetal calf serum for 6h and analyzed as above. Symbols represent means ± SEM; n=3.

Figure 3: PON2 mRNA levels are increased by DTT or tunicamycin treatment, but decreased after thapsigargin treatment. EA.hy 926 cells were left untreated or were treated with DTT (2.5 mM) **(A)**, tunicamycin (1µg/ml) **(B)**, or thapsigargin (10 nM) **(C)** for the indicated time points; in panel **(D)**, varying concentrations of thapsigargin (0.1 – 10 nM) were applied for 16h. Afterwards, total RNA was prepared and PON2 mRNA levels were determined by qRT-PCR. Symbols represent means ± SEM; n=3-4. In panels C and D, nonlinear regression was performed using an equation of one phase exponential decay.

Figure 4: Analysis of PON2 mRNA stability upon ER stress induction. EA.hy 926 cells were left untreated or were pre-treated for 4 hours with the UPR-inducing compounds DTT (2.5 mM) **(A)**, tunicamycin (0.5 µg/ml) **(B)** or thapsigargin (1 nM) **(C)**. Then, RNA synthesis was inhibited by addition of 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB; 60 µM) with prolonged presence of the UPR inducing compounds. At the indicated time points, RNA was prepared and PON2 mRNA

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expression level (decay) was determined by qRT-PCR. Symbols represent means ± SEM; n=3. Note that concentrations of tunicamycin and thapsigargin have been lowered compared to other experiments in order to prevent strong pro-apoptotic stimuli.

Figure 5: PON2 mRNA degradation is instigated by disturbed Ca^{2+}-homeostasis, but not by general activation of the UPR pathway. EA.hy 926 cells were left untreated (control) or were treated for 16h with thapsigargin (thapsi; 10 nM) **(A-C)**, Ca-ionophore A23187 (10 µM) **(D-F)**, the intracellular Ca^{2+}-chelator BAPTA/AM (BAPTA; 10 µM; 1h pre-treatment where indicated) or combinations thereof. Then, cells were lysed, RNA was prepared and mRNA levels of PON2 **(A+D)**, GRP78 **(B+E)** or spliced XBP1 **(C+F)** were monitored by qRT-PCR. **(G+H)** EA.hy 926 cells were transfected with plasmids pGL4-PON2-promoter, pGL4-PON2-promoter+5'UTR or pGL4-PON2-promoter+3'UTR. A plasmid for constitutive expression of renilla luciferase was co-transfected. Cells were treated with DMSO (0.1%; solvent control), thapsigargin (10 nM; panel **G**) or tunicamycin (10 µg/ml; panel **H**) for 16h. Then, total RNA was prepared with concomitant DNase I digestion (see Methods) and firefly luciferase mRNA levels were determined by qRT-PCR. These were normalized to renilla luciferase mRNA levels to control for transfection efficiencies. Basal expression was comparable for all constructs and was independent from the addition of 5' or 3'UTRs (not shown). **(J)** EA.hy 926 cells stably overexpressing PON2-GFP were left untreated or were treated with actinomycin D (5 µg/ml; Actino) alone or in combination with thapsigargin (10 nM) for 16h. Subsequently, total RNA was prepared and PON2-GFP mRNA levels were determined by qRT-PCR. Symbols represent means ± SEM; n=4-9. Differences were tested for statistical significance using 1-way ANOVA and Bonferronis multiple comparisons post-test; n.s. = not significant; *P<0.05; **P<0.01; ***P<0.001.

Figure 6: UPR activation by DTT or tunicamycin induces PON2 protein expression. **(A)** EA.hy 926 cells were left untreated or were treated with DTT (2.5 mM) for 4, 8 or 16 hours. Lysates (50 µg of protein) were analyzed by Western blotting using anti-PON2 and anti-α-tubulin antibodies. Results of densitometric quantification of relative signal intensities (after normalization to α-tubulin) are given below the lanes. **(B)** EA.hy 926 cells were left untreated or were treated with the indicated concentrations of tunicamycin for 8 hours and analyzed as described for panel A. Arrowhead indicates tunicamycin-induced, newly synthesized PON2 isoform-1; newly synthesized PON2 isoform-2 is not detectable after 8h treatment (see next panel). **(C)** EA.hy 926 cells were untreated (lane 1) or were treated with tunicamycin (tunica; 0.5 µg/ml) or cycloheximide (cyclo; 5 µg/ml) or both. After 8, 16, 24 or 32 h cells were harvested and lysates (50 µg) were analyzed by Western blotting using anti-PON2, anti-GRP78 and anti-α-tubulin antibodies. Arrows indicate tunicamycin-induced, newly synthesized PON2 isoforms1+2. Each blot is representative of two to four others showing similar results.

Figure 7: PON2 protein is degraded by a Ca^{2+}-dependent, calpain-mediated mechanism. **(A)** EA.hy 926 cells were solvent treated or were treated for 16h with the indicated concentrations of thapsigargin. Lysates (100 µg of protein) were analyzed by Western blotting using anti-PON2, anti-α-tubulin and anti-GRP78 antibodies. **(B)** EA.hy 926 cells were solvent treated or pre-treated with the intracellular Ca^{2+}-chelating compound BAPTA/AM (BAPTA; 20 µM) for 1h. Then, thapsigargin (10 nM) was added as indicated. After 16h, lysates were prepared and analyzed as in panel A. **(C)** Similar experiment as in panel A, but using Ca-ionophore A23187 for 16h at the indicated concentrations. **(D)** Similar experiment as in panel B, but replacing thapsigargin with A23187 at the indicated concentrations. **(E)** EA.hy 926 cells were solvent treated (DMSO) or pre-treated with the inhibitor of proteasome activity lactacystin (Lacta; 50 µM), calpain inhibitor-I N-acetyl-Leu-Leu-norleucinal (ALLN; 5 µM) or the autophagy inhibitor 3-methyladenin (3-MA, 5 mM) for 1 hour before thapsigargin (Thapsi; 10 nM) was added for another 16h. Lysates were analyzed as stated in panel A. **(F)** Naïve EA.hy 926 cells or **(G)** EA.hy 926 cells stably overexpressing PON2-iso1-GFP were left untreated or were treated with actinomycin D (5 µg/ml; Actino) alone or in combination with thapsigargin (10 nM) for the indicated time. Lysates were analyzed as stated in panel A. Results of semi-quantitative densitometric quantification for relative expression levels of endogenous PON2 (F) or PON2-GFP (G) are indicated below the lanes. Each blot is representative of 2 – 5 others with similar results. Comparable results were obtained using MG132 instead of lactacystin and cycloheximide instead of actinomycin D (panels E and F, respectively; data not shown). In panel **(H)**,

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relative to various thapsigargin concentrations (x-axis), the resulting caspase-3/7 activation (left y-axis) from Figure 1D was plotted against thapsigargin-induced decay of PON2 mRNA (from Figure 3D, inverse; right y-axis) and degradation of PON2 protein (densitometric quantification of the blot shown in panel 7A; right y-axis).

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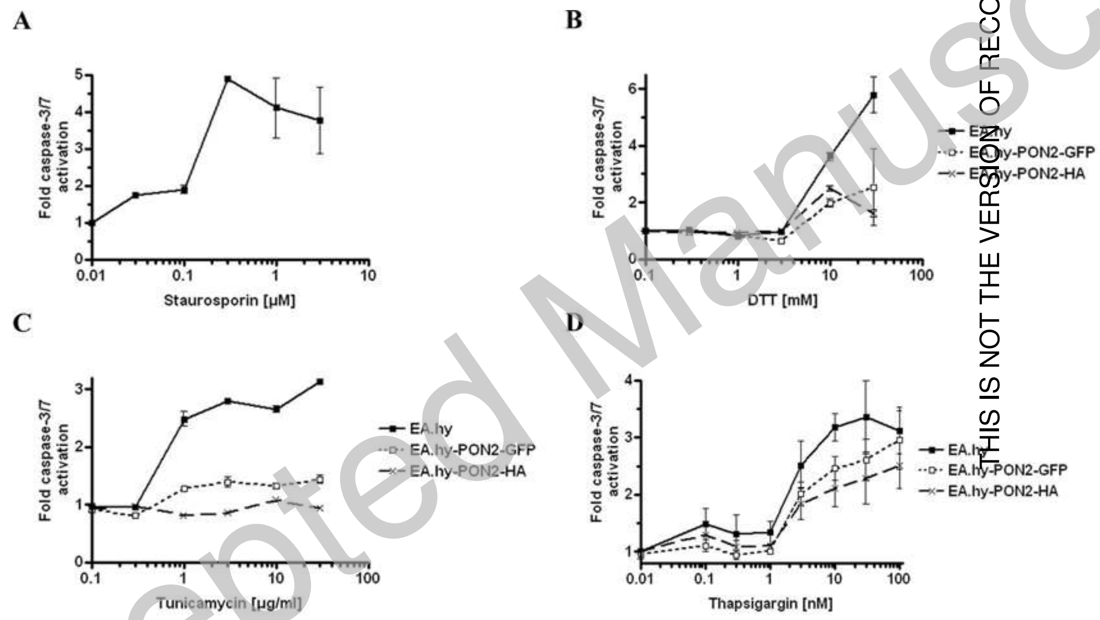

Horke et al., Figure 1

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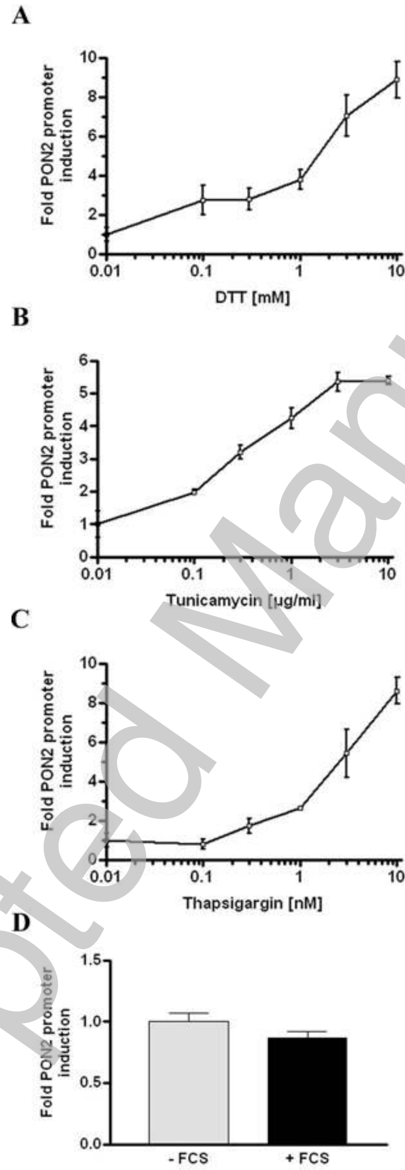

Horke et al., Figure 2

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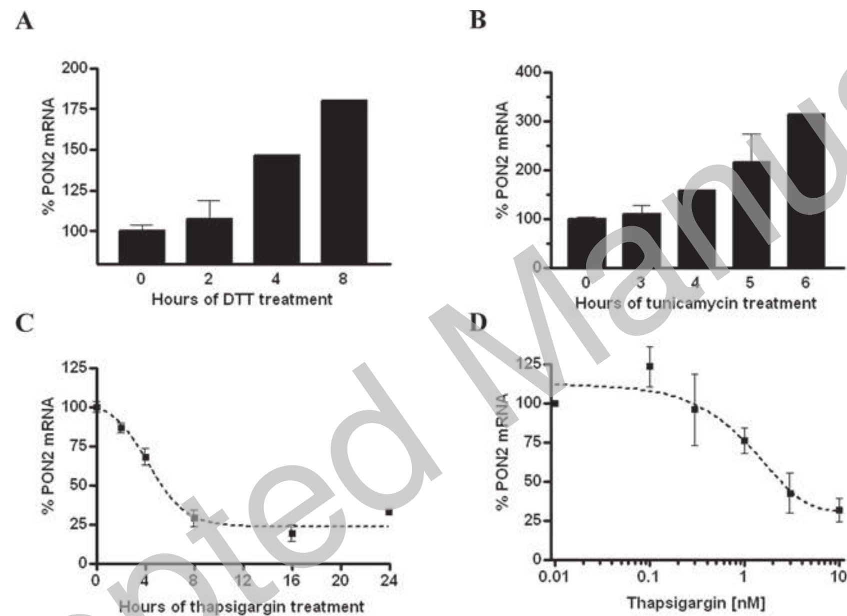

Horke et al., Figure 3

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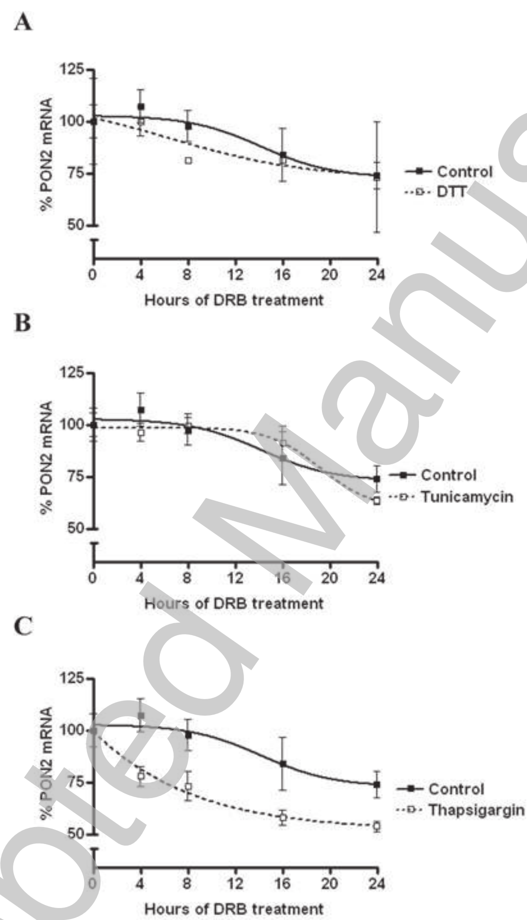

Horke et al., Figure 4

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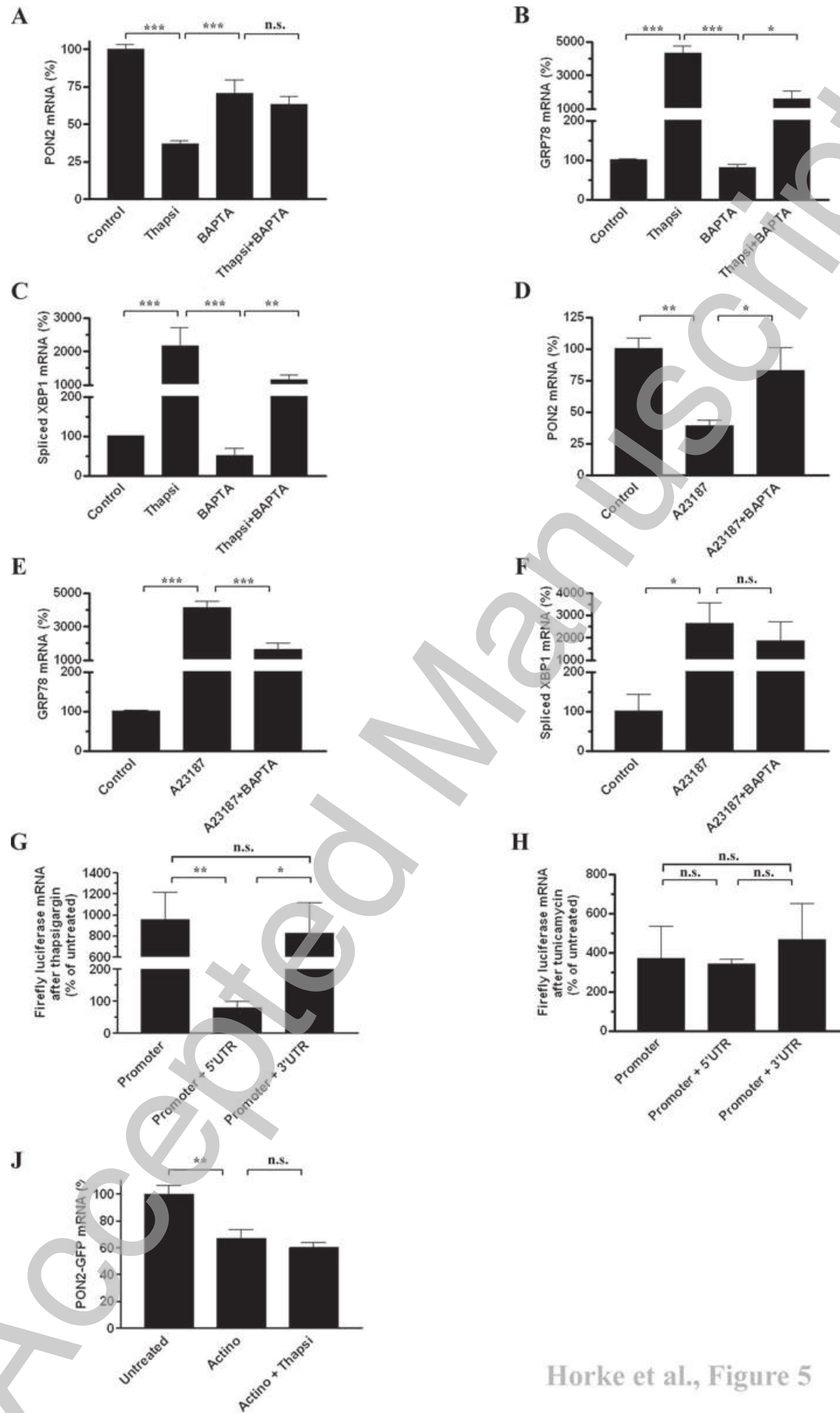

Horke et al., Figure 5

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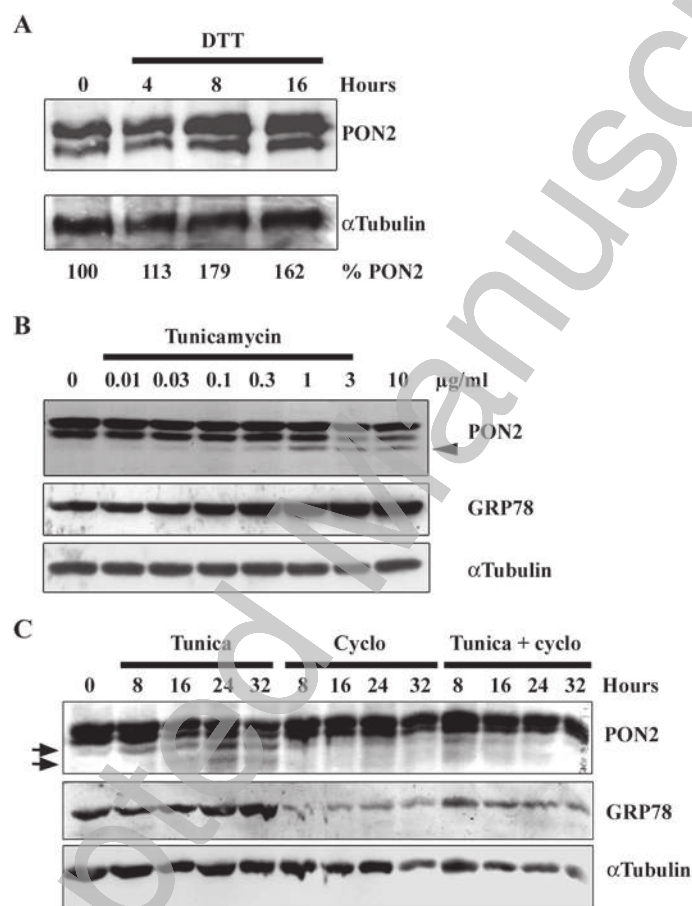

Horke et al., Figure 6

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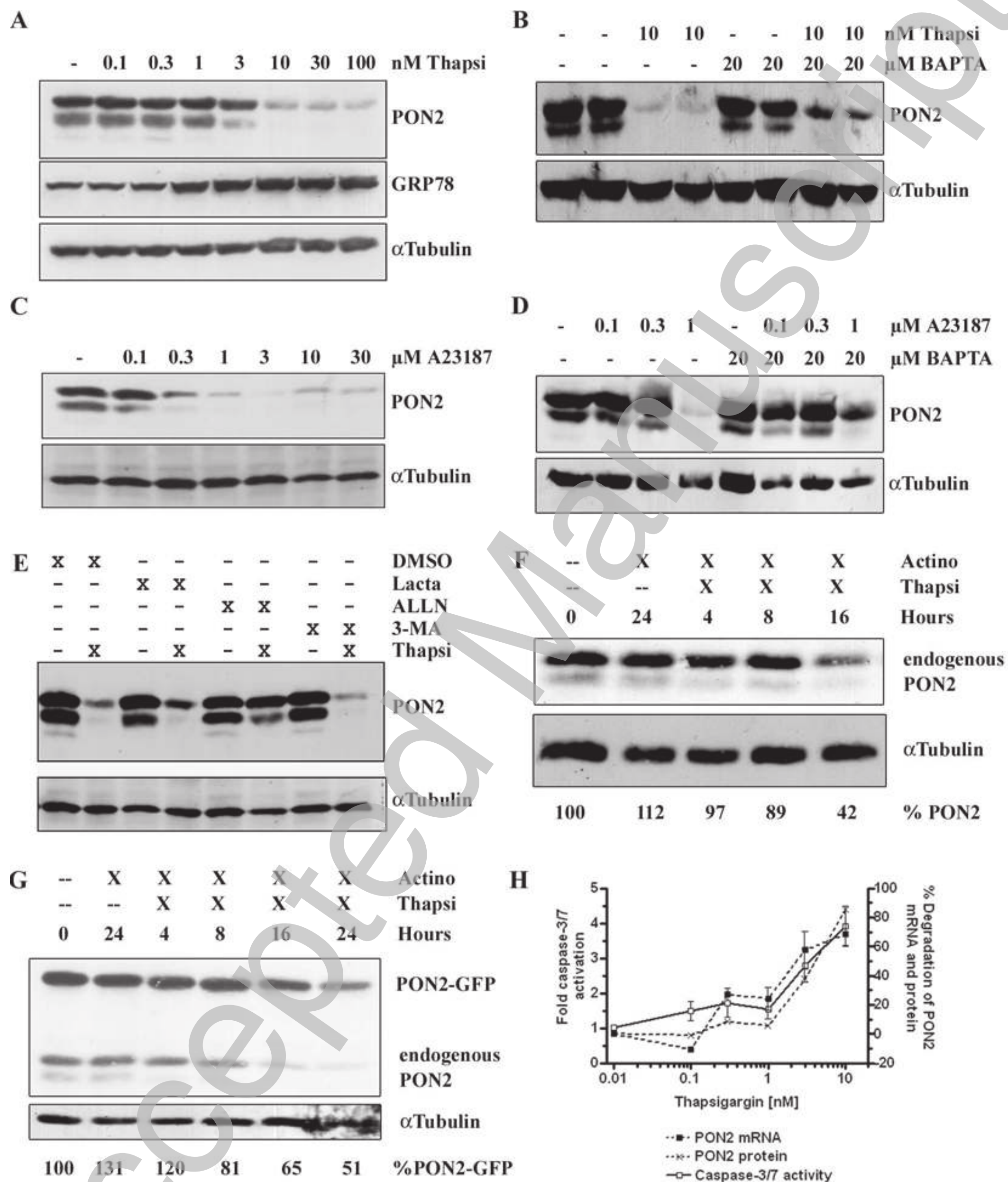

Horke et al., Figure 7

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