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Letter to the Editor

The protease inhibitor Ucf-101 induces cellular responses independently of its known target, HtrA2/Omi

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Dear Editor,

HtrA2 (also known as Omi) is a mammalian serine protease with high homology to bacterial HtrA proteases.[1,2] HtrA2 is localized in the mitochondrial intermembrane space and is released in response to apoptotic stimuli. HtrA2 can induce cell death in a caspase-dependent manner by interacting with the inhibitor of apoptosis proteins as well as in a caspase-independent manner that relies on its own protease activity.[3–6] However, mice in which the gene encoding HtrA2 has been deleted or inactivated by point mutation in the protease domain show no evidence of reduced rates of cell death, but on the contrary suffer loss of a population of neurons in the striatum resulting in a Parkinsonian syndrome.[7,8] This phenotype suggests that the predominant physiological role of HtrA2 is a cell-protective protease function, probably in the mitochondria, and not a proapoptotic action in the cytosol. Recently, two HtrA2 point mutations have been identified in Parkinson's disease (PD) patients.[9] These mutations seem to result in partial loss of proteolytic activity, possibly contributing to the aetiology of PD in these patients. Mammalian HtrA2 may therefore function *in vivo* in a manner similar to its bacterial homologues DegS and DegP, which are involved in protection against cell stress.[10,11] DegS senses unfolded proteins in the bacterial periplasm, activating a proteolytic cascade that results in the transcriptional upregulation of stress response genes. DegP degrades unfolded proteins at elevated temperatures, whereas it acts as a chaperone at low temperatures.

A mitochondrial-specific stress response has been reported to exist in mammalian cells.[12] Accumulation of unfolded proteins in the mitochondrial matrix results in the transcriptional upregulation of nuclear genes encoding mitochondrial stress proteins, via a mechanism involving the transcription factor C/EBP homologous protein (CHOP), also known as growth arrest and DNA-damage-inducible gene 153 (GADD153). Although HtrA2 is located in the intermembrane space of the mitochondria, it is possible that it might be involved in transmitting the stress signal from the matrix out of the mitochondria. Thus, we were interested in studying the induction of CHOP in response to a variety of stresses in wild-type and HtrA2-knockout mouse embryonic fibroblasts (MEFs). In addition, we used Ucf-101, a cell-permeable, furfurylidine-thiobarbituric acid compound that competitively and reversibly inhibits HtrA2 protease activity[13] (Figure 1a). Ucf-101 shows very little activity against various other serine proteases and, when tested in caspase-9 null fibroblasts, was found to inhibit HtrA2 overexpression-induced cell death.[13] On the assumption that it is a specific inhibitor of HtrA2, Ucf-101 has been employed to identify potential substrates of this protease[14,15] and to study its role in cell death.[16–19] Ucf-101 provides at least partial protection from cell death induction by cisplatin,[14,16] myocardial ischaemia and reperfusion,[17] TNFα[19] and staurosporine (our unpublished data), leading to the assumption that HtrA2 plays a role in promoting cell death following these treatments. If Ucf-101 is a specific inhibitor of HtrA2, then the effect of using Ucf-101 should be similar to the deletion of HtrA2 in MEFs.

The induction of CHOP is well studied in response to endoplasmic reticulum (ER) stress.[20,21] Tunicamycin, which inhibits N-linked glycosylation in the ER, increases the CHOP mRNA level potently in wild-type and HtrA2-knockout MEFs (Figure 1b). Thapsigargin, an inhibitor of the ER Ca^{2+}-ATPases, also induces CHOP in both cell lines (data not shown). To our surprise, however, Ucf-101 by itself induced CHOP mRNA, both in wild-type and HtrA2-knockout MEFs. After only 1 h incubation with 20 μM Ucf-101, CHOP was already induced two-fold, rising to 5- to 8-fold at 2 h. ATF3 is another transcription factor known to be stress inducible (for review see Hai and Hartman[22]): it was also induced by Ucf-101 in both wild-type and HtrA2-knockout MEFs (Figure 1c). The increase in CHOP and ATF3 mRNA levels in response to UCF-101 treatment does not seem to be cell type specific, because similar results were seen in the murine neuroblastoma cell line Neuro-2A (data not shown).

The induction of two transcription factors that are known to be responsive to stress, CHOP and ATF3, suggests that Ucf-101 might trigger activation of stress pathways in cells. This action is independent of its ability to inhibit HtrA2, as the response is seen as strongly in HtrA2-knockout as in wild-type MEFs. Therefore, we looked at the activation of various cellular stress and other signalling pathways using phospho-specific antibodies. Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) is a well-documented mechanism of downregulating protein synthesis under a variety of stress conditions.[23] The stress-activated protein kinase/Jun-N-terminal kinase SAPK/JNK is potently and preferentially activated by diverse environmental stresses and can also be phosphorylated following stimulation of a member of the germinal centre kinase family. p38 MAP kinase

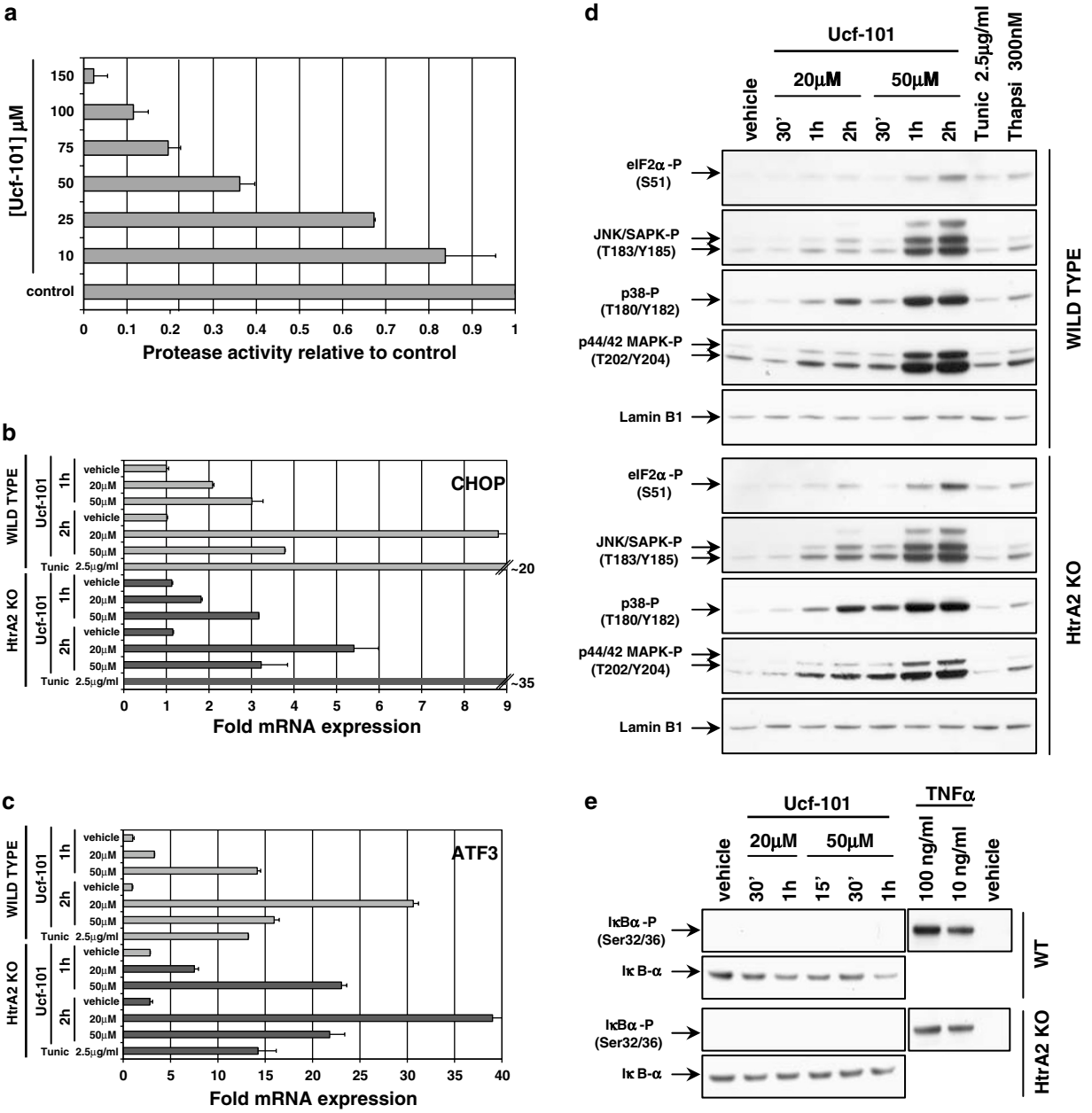

Figure 1 (**a**) Ucf-101 inhibits HtrA2 protease activity. Mature carboxy-terminal His_6-tagged Δ133HtrA2 was expressed in and purified from *Escherichia coli*. 100 nM HtrA2 were incubated with 10 μM optimal substrate (Mca-IRRVSYSF(Dnp)KK) at 30°C in PBS containing 1 mM DTT. Fluorescence was monitored on a CytoFluor multiwell plate reader (PerSeptive Biosystems). Protease activity (arbitrary fluorescence units/min) was determined by linear regression analysis of the data points corresponding to the maximum reaction rates for each assay condition. (**b**) Ucf-101 induces CHOP and (**c**) ATF3 mRNA. Total RNA was isolated from HtrA2 wild-type and knockout MEFs treated with vehicle, Ucf-101 (Calbiochem) or tunicamycin (Sigma) using the RNeasy system in combination with the RNase-free DNase kit (Qiagen). Complementary DNA was synthesized with the SuperScript III kit (Invitrogen) and used as a template for quantitative PCR analysis monitoring the real-time increase in fluorescence of SYBR Green (Applied Biosystems) on a Chromo4 detector system (MJ Research). Gene-specific primers were designed using PrimerExpress software (Applied Biosystems) and are available on request. Relative transcript levels of target genes are normalized to Actin Gamma RNA levels. (**d**) Ucf-101 induces various cellular stress pathways. Whole-cell lysate was prepared from HtrA2 wild-type and knockout MEFs following treatment with vehicle, Ucf-101, tunicamycin or thapsigargin (Sigma). Proteins were separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred onto a PVDF membrane by wet transfer. Primary antibodies were against eIF2α-phospho Ser51 (1 : 1000; Cell Signaling), JNK/SAPK-phospho Thr183/Tyr185, phospho-p38 MAPK Thr180/Tyr182, phospho-p44/42 MAPK Thr202/Tyr204 (all 1 : 1000; Cell Signaling) and Lamin B1 (1 : 1000; Santa Cruz). Secondary antibodies used were mouse-HRP, rabbit-HRP (both 1 : 2000; Amersham) and goat-HRP (1 : 5000; Pierce), and these were detected using enhanced chemiluminescence (Amersham). (**e**) NF-κB is not activated in response to Ucf-101. Primary antibodies were against phospo-IκBα (1 : 2000; Cell Signaling) and IκBα (1 : 1000; Santa Cruz). As a control for NF-κB activation, cells were treated with TNFα for 5 min

participates in a signalling cascade controlling cellular responses to cytokines and stress. Both p44 and p42 MAP kinases (ERK1 and ERK2) function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation, and are phosphorylated in response to a wide range of extracellular signals. All of these

signalling pathways are activated in a time- and concentration-dependent manner by Ucf-101 in wild-type and HtrA2-knockout MEFs (Figure 1d) and in Neuro-2A cells (data not shown). We conclude that HtrA2 is not essential for the stress responses induced by tunicamycin and thapsigargin. This may not be unexpected as these agents act primarily to cause ER, rather than mitochondrial stress. However, even at low concentrations, the HtrA2 inhibitor Ucf-101 seems to have a broad effect on the activation of cellular stress-response pathways that is independent of its ability to inhibit HtrA2 proteolytic activity. It is not clear how Ucf-101 elicits such a pronounced overall stress response.

The most important implication of these findings is that the inhibitor Ucf-101 should be used with great care and not regarded as an entirely specific inhibitor of HtrA2. Several reports have assumed that all the biological effects of Ucf-101 reflect its inhibition of HtrA2 protease activity.[14–19] As Ucf-101 can induce activation of ERK (Figure 1d), it is possible that its survival-promoting actions could be mediated by this known antiapoptotic pathway. Alternatively, moderate activation of various stress-induced pathways has been associated with cellular adaptation, or conditioning, and protection from subsequent challenge with death stimuli; so it is possible that the ability of Ucf-101 to activate these pathways might also contribute to its reported protective effects.[21] A number of agents that induce CHOP are also known to activate the NF-κB pathway. However, Ucf-101 is not able to activate this pathway: it fails to induce phosphorylation of IκBα under circumstances where TNFα gives a robust response (Figure 1e).

Is it possible that the effects of Ucf-101 in HtrA2-knockout cells are mediated by other closely related proteases, such as HtrA1, 3 or 4?[24] Although the similarity of the serine protease domains in these proteins would suggest that they may also be targeted by Ucf-101, it is likely that they have very distinct function from HtrA2. None of these proteins are known to be mitochondrial or to have consensus mitochondrial import signals; HtrA1 is reported to be secreted from cells.[25] None have been implicated in the regulation of cell death or stress signalling. If Ucf-101 is eliciting biological effects on cells through the inhibition of HtrA1, 3 or 4, it seems unlikely that it will be able to provide meaningful information about HtrA2 function, given the evident functional divergence within this family. We recommend considerable caution in the use of Ucf-101 to investigate HtrA2 function and that it should always be backed up by data from other approaches to the ablation of HtrA2 activity, such as gene knockout or RNA interference.

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