Supplementary Results & Discussion:

Results:

NMPP-elicited* IκBα*-loss is independent of HRI eIF2α-kinase activation: The concurrence of IκBα-loss and NF-κB activation and its attenuation by hemin-treatment led us to consider whether NMPP-elicited heme depletion was responsible for these findings. Conceivably, heme depletion could activate the hepatic heme-sensor HRI eIF2α-kinase resulting in the translational suppression of IκBα and consequent NF-κB-activation. Similar IκBα translational suppression and consequent NF-κB-activation has been documented upon specific activation of the other cellular eIF2α kinases (1, 2). Indeed, in parallel with IκBα-loss (Fig. S2A), NMPP treatment increased the relative ratio of phosphorylated eIF2α (eIF2αP) over basal eIF2α levels, indicating HRI activation (3). However, NMPP-treatment of cultured hepatocytes from HRI WT ($HRI^{+/+}$) and knockout (KO; $HRI^{-/-}$) mice elicited a comparable IκBα-loss, excluding causal HRI activation (Fig. 1D).

ZnPP-elicited IκBα-loss is independent of autophagy, calpain-mediated proteolysis and reactive oxygen species (ROS): Given the reported IκBα-degradation via ALD (4) and the known p62-role in this process (5), we conclusively excluded ALD in this process by documenting that knockout of the essential autophagic gene ATG5 failed to mitigate ZnPP-elicited IκBα loss (Fig. S2B). Because of possible calpain-mediated IκBα-degradation (6, 7), we also excluded its involvement by documenting that knockout of the calpain-degradation pathway (capn4 KO MEF cells) failed to abrogate ZnPP-elicited IκBα-loss (Fig. S2C). These findings coupled with those with ALD and calpain inhibitors (Fig. S4B) revealed that neither ALD nor calpain degradation played any role in this IκBα loss. Furthermore, through the use of various ROS-quenchers (Fig. S3), we excluded any possible PPIX-photoactivation and consequent ROS-mediated oxidative stress in this IκBα-loss (8).

Discussion:

The precise mechanism of the ZnPP-elicited IκB-protein sequestration is currently unknown, but some plausible mechanisms are presented. The fact that newly synthesized IκBα is particularly vulnerable to such ZnPP-sequestration implicates the involvement of its intrinsic disordered domains at a stage when it has not yet reached its mature folded state.

In search of clues on potential ZnPP-mechanisms of protein sequestration, we sought the advice of various internationally recognized expert heme/porphyrin chemists and biochemists and porphyria experts on the possible mechanistic causes. Not a single investigator was aware that ZnPP caused protein aggregation, much less IκBα-sequestration. ZnPP (along with SnPP), is actually believed to be safe and is recommended for the treatment of neonatal jaundice due to its effective inhibition of microsomal heme oxygenase (HO1), the key rate-limiting enzyme in the conversion of heme to biliverdin (9). However, *in vivo*, overproduction of protoporphyrin IX (PPIX) due to defects in ferrochelatase (congenital erythropoietic protoporphyria) or PPIX-overproduction in X-linked protoporphyria, as well as iron-deficiency or lead-poisoning induced anemias (that can exhaust iron-stores) leads to ZnPP-generation (10-12). Our findings suggest that under these conditions, these patients may easily succumb to ZnPP-elicited IκBα-sequestration and consequently unabated hepatic NF-κB-elicited activation of cytokines and chemokines. We believe, these findings would for the first-time alert physicians of this pathological potential of ZnPP and are clinically relevant not just in MDB-inducing diseases but also in clinical protoporphyrias and iron-deficiency/lead-induced anemias.

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