Published in final edited form as:

Biochem Biophys Res Commun. 2008 April 11; 368(3): 593–599.

Enhanced Ca^{2+} leak from ER Ca^{2+} stores induced by hepatitis C NS5A protein

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Abstract

The hepatitis C non-structural protein 5A (NS5A) is a Zn^{2+} -binding phosphoprotein essential for viral replication. Expression of NS5A perturbs intracellular Ca^{2+} levels by an undefined mechanism, activating transcription factors implicated in the chronic pathogenesis of hepatitis infections. Here, we demonstrate that regulated expression of NS5A enhanced the passive leak of Ca^{2+} from a subset of the endoplasmic reticulum (ER) Ca^{2+} stores. This action was not replicated by expression of the amphipathic NH_2 -membrane anchoring domain of NS5A alone, despite targeting to intracellular membranes. Depletion of the NS5A-targeted ER Ca^{2+} store was prevented under conditions of ample ATP supply suggesting compensatory Ca^{2+} ATPase activity, but observed under conditions of ATP insufficiency and in intact cells expressing NS5A.

Keywords

Ca^{2+} signaling; endoplasmic reticulum

Infection with the Hepatitis C virus (HCV) is endemic worldwide, with estimates of chronic infection in ~120-350 million individuals [1,2]. Chronic infection, which persists in ~50-80% of infected individuals, confers a significant risk of cirrhosis and hepatocellular carcinoma, and therefore represents a major healthcare burden. No protective or therapeutic vaccine currently exists and interferon-based treatment regimens lack efficacy in many patients [3,4].

HCV is an enveloped single-stranded RNA virus, characterized by a high degree of genetic variability with six major genotypes and 100 subtypes recognized [5,6]. The HCV polyprotein is co- and post-translationally cleaved by host cell and viral proteases into at least 10 different viral products, comprising structural (core & envelope), p7 and non-structural proteins (NS-2, -3, -4A, -4B, -5A & -5B). All of the non-structural proteins contribute to a ribonucleoprotein replication complex associated with the cytoplasmic face of intracellular membranes [7,8]. NS5A has attracted much interest by virtue of its putative roles in modulating host interferon responses and its anti-apoptotic and pro-proliferative activity [5,6,9]. Structurally, NS5A is a large phosphoprotein (56-58kDa, 447 amino acids) composed of an NH_2 -terminal amphipathic α -helix (amino acids 1-31, which anchors NS5A to intracellular membranes) and three cytoplasmic domains, containing sites of interaction many cellular proteins [9]. Unsurprisingly therefore, NS5A affects various cellular signaling pathways although the (patho)physiological significance of many of these interactions is unclear. For example, Gong *et al.* suggested that NS5A alters intracellular Ca^{2+} levels, leading to the induction of oxidative stress and nuclear

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translocation of NF- κ B and STAT-3 [10]. This was a very exciting finding given its consistency with previous observations that: (i) NS5A localizes to intracellular membranes including the ER [8]; (ii) mitochondrial energetics are regulated by reciprocal Ca^{2+} signaling between mitochondria and ER; (iii) reactive oxygen species (ROS, generated within mitochondria and the ER lumen) have been implicated in viral carcinogenesis and (iv) NF- κ B and STAT-3 are activated by oxidative stress and regulate cassettes of pro-survival, anti-apoptotic and proliferative genes induced in liver disease [10].

However, despite the appeal of these results, delineation of the precise mechanism by which NS5A impacted ER Ca^{2+} homeostasis to disturb intracellular Ca^{2+} was undefined and was simply ascribed to components of the ER stress response seen in cells expressing subgenomic HCV replicons [10,11]. Indeed, the only experimental evidence supporting altered intracellular Ca^{2+} levels and specifically an action of NS5A on ER Ca^{2+} homeostasis was entirely indirect: cell-permeable Ca^{2+} chelators inhibit NF- κ B and STAT-3 activation in lysates from cells transiently transfected with NS5A. Therefore, we have investigated how regulated expression of NS5A impacted ER Ca^{2+} homeostasis.

Methods

Cell culture

HEK-293 cells (ATCC, CRL-1573) were grown (37°C, 5% CO_2) in minimal essential medium (with 10% horse serum). HepG2 cells (ATCC, HB-8065) were grown (37°C, 5% CO_2) in Dulbecco's Modified Eagle's Medium (4.5 g/L glucose, L-glutamine, 10% FBS). Cells were transfected at 70% confluency (Fugene HD, Roche) and incubated for 24–48 h before imaging. The pcDNA5/FRT/TO vector (Invitrogen) was used to stably introduce NS5A genes into the genome of HEK-293 Flp-In TREx cells (Invitrogen) under the control of a tetracycline-inducible promoter. Transfection, selection and maintenance of stable cells expressing inducible constructs (induced by 1 $\mu\text{g}/\text{ml}$ tetracycline) were as directed. Nuclear injections of *Xenopus* oocytes (stage VI) were made with a Drummond Nanoject II. Construct sequences and construction methods are in Supplementary Material.

Flow Cytometry

ROS production was assessed by oxidation of dihydroethidium (DHE; Molecular Probes) to ethidium. Cells were incubated with 5 $\mu\text{mol}/\text{L}$ DHE (45mins, 37°C), washed and analyzed.

Antibody Methods

Electrophoresis and Western blotting were performed using the Invitrogen XCell NuPage Tris-Acetate and iBLOT dry blotting systems. Nitrocellulose membranes were probed with NS5A-specific antibodies (MAB8694 Millipore, 1:10000) using standard methods. For immunofluorescence, acetone-fixed cells were air dried at room temperature (RT). All antibody incubations were done in PBS with 5% BSA; washes with PBS. Fixed cells were incubated for 45min at RT with anti-NS5A antibodies (MAB8694, 1:1000).

$^{45}\text{Ca}^{2+}$ experiments

For IP_3 -evoked Ca^{2+} release experiments, cells loaded to steady state with $^{45}\text{Ca}^{2+}$ (ATP-regeneration mix (7.5mM ATP, 5mM creatine phosphate, ACK (5units/ml), or 7.5mM ATP with $^{45}\text{Ca}^{2+}$ 7.5 $\mu\text{Ci}/\text{ml}$ and FCCP, 10 μM) were diluted into MI2 (140mM KCl, 20mM NaCl, 2mM MgCl_2 , 1mM EGTA, 20mM PIPES, 300 μM CaCl_2 (free $[\text{Ca}^{2+}]$ ~200nM), pH7, 37°C) containing IP_3 before harvesting. To determine the passive Ca^{2+} release rate, cells loaded to steady state with $^{45}\text{Ca}^{2+}$ were diluted into Ca^{2+} -free medium containing thapsigargin (1 μM). Mono/multi-exponential fits compared using the partial (sequential) *F*-test.

Fluorescence microscopy and single cell Ca^{2+} imaging

Ca^{2+} transients (100 μM ATP) were imaged in HEK-293 cells loaded with 1.5 μM fura-2, for 30mins at room temperature using an Olympus IX81-based system equipped with a fura-2 filter set (Chroma), a rapid-filter switcher (Lambda DG4, Sutter) and an EM-CCD camera (C9100-02, Hamamatsu). Image analysis was performed with SimplePCI (Compix).

Results & Discussion

Expression of NS5A[1b]-GFP in HEK-293 cells resulted in the manifestation of green fluorescence in both reticular membranes, as well as punctate structures, throughout the cytoplasm (Fig. 1A). Co-expression of an ER marker (RFP-ER) confirmed ER localization of NS5A, consistent with previous studies demonstrating NS5A targeting to intracellular membranes. This localization was not an artifact of GFP-tagging, as a similar distribution was observed with untagged NS5A in immunostained HEK-293 and HepG2 cells (Fig. 1B). A similar ER expression profile was observed after transient transfection of NS5A-GFP into hepatic cell lines (e.g. HepG2) and following nuclear injection of cDNA in *Xenopus* oocytes (Fig. 1C). Finally, an 'anchor' construct (NS5A[1-31]-GFP, comprising the highly conserved N-terminal amphipathic helix essential for HCV RNA replication, also targeted to ER membranes and punctate structures (Fig. 1D). Consistent with the results of Gong *et al.* [10], we observed elevated ROS levels in DHE-based assays of NS5A and NS5A-anchor transiently transfected cell populations screened by flow cytometry (Fig. 1E). However, elevated ROS levels were also observed after overexpression of many ER-targeted constructs in transient transfection assays.

Therefore, to better resolve the effects of NS5A on intracellular Ca^{2+} stores within the same cellular background, we used an inducible expression system to compare changes between naïve (uninduced control) and NS5A-expressing (induced) cells. We developed three different inducible HEK-293 cell clones for regulated expression of NS5A[1b/2] or the NS5A anchor alone (Fig. 2), validated by both Western blotting and RT-PCR (as the NS5A antibody epitope region is lacking in the anchor construct). Anchor-specific primers revealed increases in mRNA in each of the three lines after induction (Fig. 2A). Similarly, Western analysis revealed the characteristic NS5A doublet (basally- and hyper-phosphorylated NS5A, ~56-58kDa) confirming inducible production of the full length NS5A protein. No signal was detectable in the parental cell line, or in the derived clones in the absence of tetracycline. Figure 2B shows the timecourse of NS5A production, which was maximal by 24hrs. In the absence of tetracycline, only a few cells (<1%) stained positive whereas after 48hrs incubation with tetracycline, NS5A[1b/2] expression was pervasive (Fig. 2C). Consistent with previous data (Fig. 1), NS5A localized to ER membranes. However, in contrast to what we observed in transient transfection protocols (Fig. 1E), ROS measurements showed little change after induction of NS5A (~99% of control). Therefore, consistent with other reports [12,13], we show that substantial NS5A expression is not necessarily associated with increased ROS levels.

To examine the impact of NS5A expression on ER Ca^{2+} homeostasis we performed population-based measurements on parental, anchor and the two genotype expressing cell lines. Experiments were performed to resolve (i) the rate ($t_{1/2}$) and extent of ER $^{45}\text{Ca}^{2+}$ uptake, (ii) the kinetics of passive efflux in the presence of thapsigargin (a SERCA inhibitor) and (iii) the sensitivity and magnitude of IP_3 -stimulated $^{45}\text{Ca}^{2+}$ release. As results with the two NS5A genotypes (NS5A[1b/2]) were not significantly different, we present a pooled dataset for comparison with anchor and control cells.

Active loading of ER Ca^{2+} stores with an ATP-regenerating mix revealed no significant difference in either extent (100 $\pm$ 8.4% vs 84.5 $\pm$ 13.8%, control and NS5A expressing cells) or the half-times for store loading (Fig. 3A). However, thapsigargin addition to preloaded stores

revealed a rapid initial phase of $^{45}\text{Ca}^{2+}$ efflux in NS5A induced cells compared with anchor-expressing cells or controls (Fig. 3B). Therefore, inhibition of SERCA pump function revealed different profiles of $^{45}\text{Ca}^{2+}$ leak from ER Ca^{2+} stores in the presence of NS5A. Kinetic analysis of the passive leak rate resolved that Ca^{2+} efflux from NS5A-expressing cells was best described by a bi-exponential function, with a rapid phase of efflux ($t_{1/2} \sim 10\text{s}$, $\sim 22.9\%$ of thapsigargin-sensitive Ca^{2+} store) and a slower leak rate ($t_{1/2} \sim 4\text{ mins}$, $\sim 77.1\%$ of thapsigargin-sensitive Ca^{2+} store) comparable to the mono-exponential decay kinetics observed in anchor-expressing ($t_{1/2} \sim 4\text{ mins}$, amplitude $\sim 66\%$ of steady state loading) and control cells ($t_{1/2} \sim 3.3\text{ mins}$, amplitude $\sim 65\%$ of steady state loading). These results suggest that NS5A expression induces a functionally distinct ER Ca^{2+} store ($<30\%$ of thapsigargin-sensitive Ca^{2+} store) characterized by rapid Ca^{2+} turnover.

In the presence of an ATP-regenerating system, IP_3 sensitivity was similar before ($\text{EC}_{50} = 231 \pm 39\text{nM}$) or after expression of either full length NS5A ($\text{EC}_{50} = 210 \pm 11\text{nM}$, Fig. 3) or anchor alone ($\text{EC}_{50} = 252 \pm 58\text{nM}$). Maximal concentrations of IP_3 ($\leq 100\mu\text{M}$) mobilized the majority of the intracellular Ca^{2+} store in control ($56.3 \pm 3.3\%$) and NS5A ($55.4 \pm 2.9\%$) expressing cells. Therefore, under optimized pumping conditions, NS5A does not impair Ca^{2+} cycling through ER Ca^{2+} stores (Fig. 3A) or IP_3R function (Fig. 3C).

However, in the absence of an ATP regeneration mix, the magnitude of the IP_3 -mobilizable store decreased by a greater extent in NS5A expressing cells ($39.5 \pm 2.0\%$ of total Ca^{2+} released by saturating IP_3) versus only a $\sim 10\%$ decrease in naïve cells ($51.2 \pm 2.1\%$, Fig. 3D) or anchor-expressing cells. Because the magnitude of the decrease in IP_3 -sensitive pool size after NS5A induction ($\sim 23\%$) was similar to the observed size of the rapidly leaking store revealed by NS5A expression in unidirectional efflux experiments ($\sim 23\%$, Fig. 3B), we reasoned that NS5A expression is associated with an enhanced Ca^{2+} leak from a subset of the intracellular Ca^{2+} stores revealed under conditions of ATP insufficiency.

Do these changes in functional store properties affect Ca^{2+} signals in intact cells? Using fura-2 imaging, we compared the effects of NS5A induction on Ca^{2+} signals evoked by extracellular ATP ($100\mu\text{M}$). A small decrease in the peak magnitude of the cytoplasmic Ca^{2+} signal ($\sim 17\%$, peak ratio of 4.0 ± 0.3 in control vs 3.4 ± 0.3 after NS5A expression) as well as a decrease in the overall (integral) magnitude of the response ($79.2 \pm 0.7\%$ in NS5A expressors vs control) was observed. However, there was no difference in Ca^{2+} oscillation frequency, or resting cytoplasmic Ca^{2+} levels in NS5A expressing cells (Fig. 3E). Ca^{2+} signals in NS5A-anchor expressing cells were similar to controls (Fig. 3E).

Therefore, NS5A expression is associated with compartmentalization of a discrete ER Ca^{2+} store characterized by rapid Ca^{2+} cycling (Fig. 4). Interestingly, ER structural changes have been observed in NS5A-expressing and HCV infected cells [8,14], possibly as an adaptation to promote viral replication or as a host-adaptation to preserve ER functionality. This effect is not mimicked by the NS5A anchor, suggesting that homo/hetero-typic interactions outside the amphipathic NH_2 -terminal domain are needed to enhance ER Ca^{2+} depletion. When cells are not metabolically stressed, NS5A likely causes no overt changes in intracellular Ca^{2+} homeostasis. However, enhanced ATP consumption during viral replication, or chronic impairment of mitochondrial energetics by ROS (over)production may impair compensatory ER Ca^{2+} uptake. Nonetheless, in the context of the whole polypeptide, where notably the HCV core has consistently been shown to deplete ER Ca^{2+} , disrupt mitochondrial Ca^{2+} homeostasis and evoke ROS [15-18], the individual effects of distinct HCV proteins will likely synergize to impact cellular stress signaling. Such disruption of ER Ca^{2+} homeostasis is likely an event common to many viral infections, serving to facilitate replication and persistence, as well as contributing to tissue damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Supported by NIH (NS046783) and a NSF CAREER Fellowship (JSM, 0237946).

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FIGURE 1. NS5A construct localization

(A) Localization of GFP-NS5A[1b] (left) in HEK-293 co-transfected with RFP-ER (middle) analyzed by confocal microscopy (overlay, right). *Inset*, higher magnification of GFP-NS5A labeled structures. (B) Immunofluorescence of NS5A[1b] in HEK-293 cells. (C) GFP-NS5A localization in HepG2 cells (left) and within the cortical ER of *Xenopus* oocytes (right). (D) Localization of a GFP-NS5A-anchor construct in HepG2 cells. (E) *Right*, DHE-based assay of ROS production (48hrs) in populations of NS5A-anchor (solid) and NS5A (open) transfected cells relative to non-transfected controls. *Left*, increased fluorescence in nuclei of NS5A-GFP transfected HEK-293 cells. Scalebars = 10µm.

FIGURE 2. Characterization of a NS5A inducible system in HEK cells

(A) *Top*, RT.PCR analysis using NS5A-anchor specific primers (expected product = 88bp) in parental line (pre '1' & post '2' induction), the NS5A anchor clone (pre '3' & post '4' induction) and full length NS5A variants [1b/2] clones (pre '5 & 7' & post '6 & 8' induction). *Bottom*, Western showing full length NS5A in stable clones. (B) Kinetics of NS5A production revealed by Western blotting. (C) Immunofluorescence staining of NS5A variants (1b & 2) in uninduced (-) and induced (+) HEK-293 cells visualized with an Alexa568-conjugated secondary antibody. Cell nuclei were stained with Hoechst.

FIGURE 3. ER Ca^{2+} homeostasis in NS5A-inducible cell lines

(A) Timecourse of $^{45}\text{Ca}^{2+}$ loading of intracellular Ca^{2+} stores. *Inset*, comparison of steady state ('extent') and half-time for loading in control (solid) and NS5A-induced cells (open). (B) Thapsigargin-induced leak from ER Ca^{2+} stores as a function of time in control (square), NS5A (circle) and NS5A-anchor (triangle) expressing clones. *Inset*, half-times for Ca^{2+} efflux for single (control, anchor) and bi-exponential (NS5A) curve fits. (C & D) IP_3 -evoked Ca^{2+} release from control (square) and NS5A-expressing (circles) cells in the presence (solid) or absence (open) of an ATP regeneration mix. (E) Averaged single cell Ca^{2+} signals in cells expressing NS5A (red) and NS5A-anchor (blue) evoked by ATP (100 μM).

FIGURE 4. NS5A action on ER Ca²⁺ stores

Top, ER luminal Ca²⁺ content represents a balance between ATP-driven 'uptake' and 'release' components. *Bottom*, NS5A expression is associated with induction of a functionally distinct, ER Ca²⁺ store (blue dashed line) characterized by an enhanced passive Ca²⁺ leak (blue arrow), counteracted by enhanced SERCA activity when ATP supply is not limited. Increased ATP consumption or mitochondrial impairment leads to ATP depletion and decreased Ca²⁺ content of the NS5A-induced store.