Redox modulation of hepatitis C virus replication complex is calcium dependent

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This work was supported by grants from National Institutes of Health and funds from the University of California, Merced.

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Running Title: H_2O_2 and calcium on HCV

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Abstract

Reactive species and perturbation of the redox balance have been implicated in the pathogenesis of many viral diseases, including hepatitis C. Previously, we made a surprising discovery that concentrations of H_2O_2 that are non-toxic to host cells disrupted hepatitis C virus (HCV) replication complex (RC) in Huh7 human hepatoma cells in a manner that suggested signaling. Here, we show that H_2O_2 and interferon gamma have comparable effects on the HCV subgenomic and genomic RNA replication in Huh7 cells. H_2O_2 induced a gradual rise in the intracellular calcium concentration ($[Ca^{2+}]_i$). Both rapid and sustained suppression of HCV RNA replication by H_2O_2 depended on this calcium elevation. The peroxide-induced $[Ca^{2+}]_i$ elevation was independent of extracellular calcium and derived, at least in part, from the endoplasmic reticulum. Likewise, the suppression of HCV RC by H_2O_2 was independent of extracellular calcium but required intracellular calcium source. Other agents that elevated $[Ca^{2+}]_i$ could also suppress HCV RC, suggesting that calcium elevation might be sufficient to suppress HCV RNA replication. In conclusion, oxidants may modulate HCV RC through calcium. Effects on the infectivity and the morphogenesis of HCV remain to be determined. These findings suggest possible regulatory roles of redox and calcium signaling during viral infections.

Keywords: calcium, endoplasmic reticulum, glucose oxidase, glutathione, hepatitis C virus, hydrogen peroxide, replication, replicon, thapsigargin

Introduction

Reactive oxygen species (ROS) and other reactive species are products of normal cell metabolism [1]. The synthesis of reactive species, however, is heightened during inflammation [2]. The oxidative stress that ensues is believed to help fight off various infections, for example, by inflicting oxidative injury to the invading pathogens. In fact, increased levels of reactive species and decreased levels of antioxidant molecules have now been documented in many viral diseases. However, ROS also affect and participate in signaling [3-5] and, in this manner, may have other effects on viruses. For example, ROS can negatively regulate hepatitis B virus (HBV) replication in liver cells without affecting the cell metabolism [6] but enhance the replication of human immunodeficiency virus (HIV) by activating nuclear factor kappa B (NFκB) [7]. Likewise, sublethal and biologically relevant concentrations of ROS and, in particular, H_2O_2 have been found to rapidly suppress hepatitis C virus (HCV) RNA replication in Huh 7 human hepatoma cells in a manner that suggested signaling [8].

Hepatitis C virus (HCV) is a positive-sensed, single-stranded RNA virus of the *Flaviviridae* family [9]. HCV replication is mediated by NS5B and other nonstructural proteins that comprise the replication complex (RC). HCV infection is associated with increases in various markers of oxidative stress in patients [10-13]. In addition to chronic inflammation, iron overload and some of HCV proteins may help increase the oxidative burden [10, 14-21]. Increased levels of ROS and nitrogen species are suggested to enhance the pathogenesis of HCV by promoting DNA damage and steatosis [19, 22]. In addition, H_2O_2 has been found to suppress hepatitis C virus (HCV) RNA replication in cell culture [8]. This suppression was accompanied by a loss of HCV proteins and HCV-replicating activity that co-fractionated with the Golgi membranes. There was no change in host cell viability, overall intracellular redox status,

housekeeping gene expression, ribosomal RNA synthesis, or subcellular distribution of albumin with the peroxide treatment, suggesting that the suppression was rather specific to HCV.

Therefore, in this study, we tested whether ROS rapidly suppressed the activity HCV replication complex through redox signaling, to understand the biological consequence of increased oxidative stress in hepatitis C. The effect of H_2O_2 on HCV RNA replication is also compared with that of interferon gamma (IFNγ). Interestingly, various HCV proteins have been suggested to modulate calcium metabolism [17, 23, 24]. HCV p7 protein may act as a calcium channel [24]. As sublethal concentrations of oxidants can initiate calcium signaling [25-28], we examined the possible involvement of calcium in the modulation of HCV RC by H_2O_2. Our data indicate that H_2O_2 causes a gradual rise in the intracellular calcium concentration ($[Ca^{2+}]_i$) in Huh7 cells, at least in part, by releasing calcium from the endoplasmic reticulum. Buffering this calcium completely removes the oxidative suppression of the RC. Other agents that elevate intracellular calcium concentration can elicit a similar suppressive response. These data highlight the importance of redox and calcium homeostasis during HCV infection. Possible implications of these findings are discussed.

Materials and Methods

Cell Culture and Electroporation of HCV RNA – Huh7 human hepatoma cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA), supplemented with 10 % fetal bovine serum (FBS, Invitrogen), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in 5 % CO_2 incubator. SgPC2 cells are G418-selected, pooled Huh7 cell clones that support the continuous replication of subgenomic HCV replicon of genotype 1b [8, 29] (Genbank accession no. AJ242652) that carries a S1179I adaptive mutation within the NS5A region [30]. SgPC2 cells were maintained in the above medium, supplemented with 0.5 mg/ml of G418 (Invitrogen). G418 was removed at least one day prior to cell treatments.

For transient replication experiments, the hybrid genomic plasmid [8] was linearized with XbaI and used to synthesize genomic HCV RNA, as previously described [8]. Then, 5 x 10^6 Huh7 cells were rinsed with DMEM, mixed briefly with 10 - 20 μg of the subgenomic replicon RNA in 0.4 ml of DMEM, and then electroporated at 220 volts and 975 μF. Then, 1 million cells were seeded onto 60-mm cell culture dishes in DMEM containing 10% FBS. Medium was changed daily and, after 2 - 4 days, pre-incubated with BAPTA-AM or vehicle control alone for 1 hr. Then, these cells were exposed to H_2O_2 for 24 hrs, and the amount of the HCV RNA was analyzed by Northern blot (see below). A negative control RNA (H77c) [30, 31] has been shown not to replicate under these conditions [8].

$[Ca^{2+}]_i$ Measurement -- Huh7 and SgPC2 cells were grown as a monolayer on glass coverslips, and $[Ca^{2+}]_i$ was monitored, as previously described [32]. Briefly, on the day of the experiment, cells were washed in Krebs-Ringer phosphate buffer (KRPH, pH 7.4 containing 1.0 mM $MgSO_4$,

1.3 mM $CaCl_2$, 10 mM HEPE, 5 mM glucose, 125 mM NaCl, 5 mM KCl, and 10 mM sodium phosphate) and subsequently, loaded with 10 - 20 μM indo-1AM (70 μl/coverslip, Molecular Probes, Inc., Eugene, OR), containing 10 μM probenecid (Molecular Probes) for 30 min at 37°C. The attached cells were washed and bathed in KRPH/probenecid at 4°C or room temperature until further analysis. Then, coverslips were individually submerged into KRPH, pre-equilibrated inside the F2000 fluorimeter (Hitachi) in darkness for 3 min, and $[Ca^{2+}]_i$ was monitored continuously at 37°C, with excitation at 380 nm and emission at 400 and 450 nm. H_2O_2 and other agents were injected at different time points, as indicated in Results. $[Ca^{2+}]_i$ was calculated, as described [32]. R_{max} was determined by adding 8 μM digitonin and R_{min}, with 10mM ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA). The baseline, which represented unstimulated cells scanned over time, was plotted along with other measurements or subtracted from the other plots. Cells were serum-depleted, one day before the experiment, by incubating them overnight in DMEM plus 0.5 % FBS. The serum-depletion or the absence of serum in the cell incubation buffer did not affect the suppression of HCV replication in SgPC2 cells by H_2O_2 (see Results). For studies that required the absence of extracellular calcium, KRPH was prepared without $CaCl_2$, and 0.1mM EGTA was added. Then, $CaCl_2$ was added prior to determining the Rmax for each run.

Enzymatic generation of H_2O_2 by glucose oxidase (GO) -- Cells were incubated with various concentrations of GO (SigmaAldrich, St. Louis, MO) in the cell culture medium (DMEM, high glucose -- already contains 25 mM β-D(+)-glucose which is needed for the reaction), supplemented with 10 % fetal bovine serum, for 24 hrs [33]. Then, cells were washed with phosphate buffered saline (PBS) and harvested, as indicated in Results. One hundred thirty to

200 U/ml of catalase (SigmaAldrich) were added to confirm the role of exogenous H_2O_2 in the modulation of HCV replication by GO.

^{3}H labeling of newly synthesized HCV RNA -- One million SgPC2 cells were pretreated with 2 μM BAPTA-AM or DMSO alone for 1 hr and then, treated with 0 or 100 μM H_2O_2 in the presence of 100 μCi of ^{3}H-uridine (35 - 50 Ci/mmol; MP Biomedicals, Irvine, CA) and 4 μg/ml actinomycin D (SigmaAldrich, St. Louis, MO) for 5 to 6 hrs, and then lysed for the RNA isolation. RNA was analyzed on a 1 % formaldehyde agarose gel, which was then treated with 1M sodium salicylate for 20 – 30 min for fluorography. The RNA gel was also stained with ethidium bromide to visualize rRNA bands to serve as loading controls.

Northern blot analysis -- Total RNA was extracted from cells, using Trizol reagent (Invitrogen), following the manufacturer's protocol. Five to 20 μg of RNA were then subjected to Northern-blot analysis using a ^{32}P-labeled double-stranded DNA probe, prepared from nt. 3669 to 6016 of the subgenomic replicon. For these and all other experiments, RNA and protein bands were quantified with a phosphoimager (Cyclone, Perkin Elmer) or by densitometry, using SigmaScan (Jandel Scientific) or IS2000R (Kodak). The intensities of the bands of interest were normalized against the control glyceraldehyde 3-phosphodehydrogenase (GAPDH) mRNA.

Western blot analysis of HCV NS5A -- Cells were sonicated in 2x Laemmli buffer and total protein samples were analyzed for HCV NS5A level by western blot, using monoclonal anti-NS5A antibodies (Biodesign) and the corresponding horse radish peroxidase-conjugated

secondary antibodies. Chemiluminescent images were processed with ECL kit from Amersham Pharmacia and analyzed with IS2000 Gel Imaging system (Kodak).

In vitro HCV replication assay -- *In vitro* replication assay was carried out according to the protocol of Ali et al. [34], with slight modifications. Briefly, SgPC2 cells were grown to approximately 50 – 60 % confluence in 100-mm dishes. Then, the cells were treated for 30 min with various agents, and cytoplasmic lysates were prepared by gently lysing the cells in the incomplete replication buffer (prepared without dithiothreitol) immediately after treating cells with lysolecithin-containing washing solution (150 mM sucrose, 30 mM HEPES pH 7.4, 33 mM ammonium chloride, 7 mM KCl, 4.5 mM magnesium acetate, 250 μg/ml lysolecithin) for 1 – 2 minutes. Twenty to 50 μCi of α-^{32}P CTP (3,000 Ci/mmol) and 1 - 2 μg of actinomycin D were added to 70 μl of these cytoplasmic lysates, and the replication reaction was allowed to proceed for 1 hr at 30°C. Then, RNA was isolated by phenol:chloroform extraction and ethanol precipitation. The ^{32}P-labeled transcripts were then analyzed on a 1 % formaldehyde agarose gel which was subsequently washed with H_2O, dried, and analyzed with a phosphoimager.

Determination of intracellular glutathione (GSH) and ATP concentrations – Intracellular GSH concentration was determined, using GSH recycling assay [35], and expressed as nmol/mg protein. ATP level was measured, using Somatic Cell ATP Assay kit from Sigma-Aldrich, as previously described [32]. Protein level was determined with bicinchoninic acid kit (Pierce Biotechnology, Inc., Rockford, IL).

Mitochondrial membrane potential and apoptosis – Mitochondrial membrane potential was measured with JC1, using Guava cytometer (Guava Technologies, Inc.). Apoptosis was also monitored with annexin V-FITC, with 96-Nexin kit from Guava Technologies, Inc.

NS5B RdRp assay – Full length NS5B protein was purified from *E.coli*, as previously described, and was used to catalyze *de novo* RNA synthesis *in vitro* [36].

Statistics – Data are presented as mean ± SEM (standard error of the mean) of several independent experiments. Data were analyzed, using Student's t-test or one-way analysis of variance (ANOVA). P value ≤ 0.5 was considered significant. All experiments were repeated 2 – 6 times

Results

First, we compared the HCV RNA-lowering activity of H_2O_2 with that of interferon gamma (IFNγ) which is known to inhibit HCV RNA replication in Huh7 human hepatoma cells [37]. Huh7-derived SgPC2 cells, which support the continuous replication of Con1 subgenomic HCV RNA [8, 29, 30], were incubated with H_2O_2 or IFNγ for 24 hrs and analyzed for the HCV RNA level by northern blots. As shown in Figure 1, IFNγ decreased the HCV RNA level dose-dependently, to about 15 % of control level at the concentration of 100 U/ml. H_2O_2 also suppressed HCV RNA level dose-dependently with an IC50 of about 25 μM, and 100 μM H_2O_2 was as effective as 100 to 2,500 U/ml of IFNγ at suppressing the HCV RNA level (Fig. 1). Higher concentrations of H_2O_2 were not tested to stay within the sub-toxic range. Unlike H_2O_2, which showed a significant suppression of HCV RNA replication within 30 min of treatment [8] (also, see Fig. 2), however, IFNγ treatment had no appreciable effect on HCV RNA replication after 30 min (data not shown). Therefore, H_2O_2 showed a faster onset of suppressive activity than IFNγ.

H_2O_2 has been shown to elevate intracellular calcium concentration in primary hepatocytes [27, 38]. To elucidate the mechanism underlying the suppression of HCV replication by H_2O_2, we examined whether H_2O_2 induces calcium elevation in SgPC2 cells. SgPC2 cells were loaded with indo 1-AM and monitored for changes in $[Ca^{2+}]_i$. H_2O_2 caused a gradual rise in $[Ca^{2+}]_i$ (Figure 2A) that was dose-dependent and persisted for at least 2 hrs (data not shown). The calcium response of SgPC2 cells was comparable to that of naïve Huh7 cells (data not shown).

To test whether this elevation of $[Ca^{2+}]_i$ played a role in the oxidative suppression of HCV replication, we used 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis

acetoxymethyl ester (BAPTA-AM), which effectively buffered $[Ca^{2+}]_i$ in these cells (Figure 2A). Then, SgPC2 cells, either pretreated with BAPTA-AM or control vehicle alone, were incubated with ^{3}H-uridine and actinomycin D, with or without H_2O_2 for ~ 6 hrs, to look for changes in the rate of appearance of newly synthesized HCV transcripts. H_2O_2 significantly decreased the amount of the HCV RNA that was synthesized during these 6 hrs (Figure 2B). Interestingly, BAPTA-AM had no significant effect on the HCV RNA replication by itself but completely removed the suppression of HCV RNA replication by H_2O_2 (Figure 2B).

To determine the time course of calcium dependence of this suppression by H_2O_2, control and BAPTA-pretreated cells were exposed to H_2O_2 for 30 min or 3 hrs, and the cytoplasmic lysates that contained HCV RC were analyzed for *in vitro* HCV RNA replication. H_2O_2 treatment led to the suppression of *in vitro* HCV replication (Figure 2C). BAPTA-AM removed this suppression at both time points tested (Figure 2C), although the protective effect of BAPTA-AM was more variable at 30 min. Similarly, buffering $[Ca^{2+}]_i$ inhibited the peroxide-induced decrease in the total HCV RNA level, as analyzed by northern blot, 24 hrs after the H_2O_2 treatment (Figure 2D). BAPTA-AM also prevented H_2O_2-induced reduction in the genomic HCV RNA level in these cells (Figure 2E). Therefore, the inhibition of $[Ca^{2+}]_i$ elevation resulted in the loss of both rapid and sustained suppression of HCV RNA replication by H_2O_2.

To continue to study the effect of sustained oxidative stress [10-13] and the continuous generation of H_2O_2 by NAD(P)H oxidase of Kupffer cells and polymorphonuclear cells [39-41] during chronic hepatitis C, GO plus glucose, which continuously generates H_2O_2 enzymatically [33], was used. SgPC2 cells were exposed to different concentrations of GO and analyzed for changes in the HCV RNA level. As shown in Figure 3A, GO dose-dependently suppressed HCV replication. Exogenous catalase attenuated the suppression by GO, confirming that the

suppression was induced by the extracellularly generated H_2O_2 (Figure 3B). BAPTA-AM was also able to alleviate the suppressive effect of GO on HCV RNA (Figure 3B). HCV NS5A protein showed similar changes with GO, BAPTA, and catalase as the HCV RNA (Figure 3C). Therefore, both bolus and continuous H_2O_2 treatment suppressed HCV replication through elevated $[Ca^{2+}]_i$.

Next, we continued to examine whether endogenous antioxidants could then enhance the activity of HCV RC by antagonizing or reversing the suppression by oxidants. Decreasing total intracellular glutathione (GSH) concentration by about 75 % (Fig. 3A) with *L*-buthionine *S,R*-sulfoximine (BSO), an irreversible inhibitor of GSH biosynthesis [42], was sufficient to decrease the HCV RNA level and enhanced the lowering of HCV RNA by H_2O_2 (Figure 4B). Moreover, this suppression could be overcome with BAPTA-AM (data not shown). Therefore, GSH may enhance HCV RNA replication, and decreasing GSH, promote the suppression of HCV RNA replication by oxidants.

In order to understand the molecular events leading to calcium elevation and subsequent suppression of HCV RNA replication, we next determined the source of the elevated calcium in response to H_2O_2. H_2O_2 caused similar elevation of $[Ca^{2+}]_i$ in the presence and absence of extracellular calcium (Figure 5A). Therefore, H_2O_2 was not likely to elevate $[Ca^{2+}]_i$ by causing an influx of extracellular calcium but rather, by releasing calcium from internal calcium store(s). Endoplasmic reticulum (ER) is one of the major intracellular calcium stores and plays an important role in calcium signaling. To examine whether the peroxide released calcium from the ER, SgPC2 cells were first exposed to H_2O_2 and then, treated with thapsigargin (TG). TG passively depletes the calcium pool in the ER by inhibiting Ca^{2+}/ATP'ase that normally replenishes this calcium pool [25, 43]. As increases in the $[Ca^{2+}]_i$ can trigger the influx of

extracellular calcium [25], experiments were conducted in the absence of extracellular calcium to focus on the intracellular events. As expected, TG caused a transient rise in $[Ca^{2+}]_i$ (Figure 5B). Pre-incubating cells with H_2O_2 significantly decreased the magnitude of the calcium response to TG (Figure 5B). Similarly, pre-treating cells with TG diminished the H_2O_2-induced rises in $[Ca^{2+}]_i$ (Figure 5C). Thus, H_2O_2 increased $[Ca^{2+}]_i$ at least partially by triggering the release of calcium from the ER, as previously reported with other cell types by others [26, 27]. Possible effects of H_2O_2 on other intracellular calcium stores or the Ca^{2+}/ATP'ases in the plasma membrane, however, have not been ruled out. For example, H_2O_2 might inhibit the calcium extrusion by Ca^{2+}/ATP'ases in the plasma membrane and/or the mitochondrial calcium uptake to prolong the cytosolic calcium elevation, following the ER calcium release. Note that the TG-induced rises in the $[Ca^{2+}]_i$ were not different in SgPC2 versus naïve Huh7 cells under our experimental conditions.

Consistent with data in Figure 5, neither the serum depletion nor the absence of extracellular calcium had significant effects on the suppression of HCV replication by H_2O_2 (Figure 6). When both intracellular and extracellular calcium were depleted by pre-incubating cells with ionomycin, a calcium ionophore, in a calcium-free buffer, however, the suppressive response to H_2O_2 was significantly alleviated (Figure 6). These data further confirmed that the inhibition of HCV replication by the peroxide required calcium release from an internal store.

As previously discussed, calcium release from the ER can lead to mitochondrial calcium uptake, leading to mitochondrial dysfunction and apoptosis. To examine whether H_2O_2 triggered mitochondrial dysfunction to result in the rapid inhibition of HCV replication, we first measured the cellular ATP content. H_2O_2 did not cause any decline in the total ATP content after 30 min or 24 hrs of treatment (125.4 ± 0.3 % and 97.3 ± 8.25 % of untreated control at 30 min and 24

hrs, respectively; $P > 0.05$). H_2O_2 also had no significant effect on the mitochondrial membrane potential or apoptosis, either, as measured with JC-1 and annexin V-FITC staining (data not shown). These findings are consistent with our previous observation that the concentrations of H_2O_2 we employed did not cause any decrease in cell viability, reduced GSH concentration, or host rRNA synthesis in these cells [8]. Therefore, even though HCV infection has been associated with various mitochondrial abnormalities [19, 44, 45], H_2O_2 did not appear to inhibit HCV replication by inducing mitochondrial permeability transition in the hepatocytes at the concentrations used in our study.

These experiments so far indicated that the oxidative suppression of HCV replication was almost entirely calcium dependent. To determine whether the elevation of $[Ca^{2+}]_i$ was not only necessary but also sufficient to cause such suppression, we examined whether other ways of increasing $[Ca^{2+}]_i$ had similar inhibitory effects on HCV. As H_2O_2 released calcium from the ER (Figure 5), SgPC2 cells were first treated with TG for 15 min and analyzed for changes in the *in vitro* replication potential of HCV. As shown in Figure 7A, TG decreased the *in vitro* HCV RNA replication. Ionomycin elicited similar suppressive effects on HCV replication (Figure 7A). Note that these experiments were focused on early events (i.e., within 15 to 30 minutes of treatment), as these agents can be toxic and their long term effects would be difficult to interpret. In addition, adding $CaCl_2$ directly to the cytoplasmic lysates, prior to *in vitro* replication assays, could decrease HCV RNA replication (Figures 7B). Therefore, increasing $[Ca^{2+}]_i$ might not only be necessary for the oxidative suppression of HCV RC but also, sufficient to inhibit HCV RC. Consistent with this conclusion, BAPTA-AM tended to increase the basal HCV RNA level, particularly with a prolonged incubation (e.g., 24 hrs, Figures 2D and 2E). This might be

explained by fluctuations in $[Ca^{2+}]_i$ and/or ROS generation during cell metabolism and those induced by HCV [17, 23, 24].

Previously, several divalent cations (Fe^{2+} and Zn^{2+}) had been shown to inhibit HCV RNA-dependent RNA polymerase (NS5B) activity [46-49]. However, calcium only marginally suppressed the *in vitro de novo* RNA synthesis by NS5B at 0.1 and 1 μM concentrations (Figure 7C) and enhanced the polymerase activity at higher concentrations. Therefore, NS5B did not appear to be the calcium-binding target molecule that rendered HCV RC redox- and calcium-sensitive, at least under these conditions.

Discussion

In this report, we show that H_2O_2 suppresses HCV RC through calcium elevation. Previously, oxidants have been reported to elevate $[Ca^{2+}]_i$ by multiple mechanisms, including an influx of extracellular calcium [27], release of calcium bound to annexin VI [28], and release of calcium from the ER [50] (For review, see reference [25]). In this study, extracellular calcium had no significant effect on the peroxide-induced calcium elevation. TG decreased peroxide-induced calcium elevation and *vise-versa*, indicating that the peroxide-induced calcium elevation derived, at least in part, from the ER. It is interesting that this release of calcium from the ER did not trigger capacitative calcium entry in these cells. We also found that H_2O_2 did not increase IP_3 level in our study (unpublished observation). The mechanism by which H_2O_2 releases the calcium pool in the ER may thus involve increased sensitivity of inositol 1,3-triphosphate (IP_3) receptors to IP_3 and oxidation of sulfhydryl moieties on IP_3 receptors, as proposed by others [50, 51]. The peroxide-induced calcium elevation, however, did not follow a transient pattern, which would be expected from an ER-mediated calcium release, and the

present data do not preclude the possible effect of H_2O_2 on other components of calcium metabolism, such as the inhibition of Ca^{2+}/ATP'ases in the plasma membrane and inhibition of calcium uptake by the mitochondria. It should be noted that previously, nonstructural proteins of HCV have been suggested to induce ER stress with a subsequent release of calcium from the ER that would increase ROS generation by mitochondria [52]. In this study, however, we did not detect any significant change in the basal $[Ca^{2+}]_i$ or the magnitude of thapsigargin-induced calcium elevation in replicon versus naïve Huh7 cells, suggesting that the calcium pool in the ER remained largely unaffected by the HCV nonstructural proteins. In addition, our data suggest that mitochondrial permeability transition did not occur with the peroxide treatment.

How calcium affects the activity of HCV RC is yet unknown. The decrease in NS5A protein level (Figure 3C) after 24 hrs of GO exposure was most likely a result of decreased HCV replication than the cause, as the protein level did not change at 30 min of H_2O_2 treatment [8]. In our previous study, the oxidative suppression of HCV replication was not associated with a change in the stability of HCV RNA transcript or decreased amounts of HCV proteins and the RNA template. Instead, the suppression was associated with the disappearance of HCV replicating activity that co-fractionated with the Golgi membranes [8]. Our findings are consistent with findings of Serafino *et al.* and Aizaki *et al.* [53, 54]. Lipid rafts and membranous webs have also been implicated as important sites of HCV RNA replication [54-56]. It is possible that calcium binds to host and/or viral proteins to cause a disruption of HCV RC in the membranes. Experiments are underway to identify the calcium effector protein(s) that mediate(s) the suppression of HCV replication by H_2O_2. Interestingly, although Ca^{2+} dose-dependently suppressed HCV replication when directly added to *in vitro* reactions (Fig. 7B), whether the magnitude of calcium elevation always correlates with the level of suppression of

HCV replication *in vitro* and *in vivo* (Fig. 2 and 7), is not clear. Similar disparity between *in vivo* versus *in vitro* concentrations of calcium required for various protein functions has been observed with well-characterized calcium binding proteins like calpain and calpastatin [57], and might be explained by highly localized calcium transients inside the cells, perturbation of normal cell metabolism and signaling processes in the *in vitro* studies, relatively high affinity of the calcium effector protein(s) to calcium (e.g., than indo 1-AM, which only binds to free calcium), and the existence of multiple calcium effector protein(s) with different calcium-binding activity. Surprisingly, unlike Fe^{2+} and Zn^{2+}, which have been shown to suppress NS5B polymerase activity, we found that calcium did not significantly suppress the polymerase activity, at least *in vitro* (Figure 7C). The enhancement we saw with 100 μM calcium concentration is intriguing but its biological relevance is questioned. Preliminary studies using multiple inhibitor compounds suggested that at least two of the well-characterized calcium-binding proteins, calpain and calmodulin, are not involved. During the course of this investigation, Waris *et al*. reported a suppression of HCV RNA replication by NFκB-mediated induction of cyclooxygenase-2 gene expression [58]. However, this mechanism cannot explain the rapid suppression of HCV RC we observed within 15 – 30 min. Thus, while finding the ultimate target molecule(s) might reveal potential targets for antiviral therapy, the identity of the calcium-binding target molecule remains elusive. Importantly, TG caused a more transient elevation in $[Ca^{2+}]_i$ than H_2O_2, which was followed by a decline; likewise, TG elicited a transient suppression of HCV replication, followed by a rebound, with prolonged incubation (data not shown). Therefore, while looking for potential antiviral targets, it will be important to consider the kinetics of $[Ca^{2+}]_i$ modulation as well as the kinetics of the observed antiviral response.

In the previous study, NAC, a precursor of cysteine for GSH biosynthesis and a thiol reductant, was able to partially antagonize the oxidative suppression of HCV RNA replication [8]. In the present study, we also showed that decreasing intracellular concentration of GSH, the most abundant intracellular thiol reductant, was sufficient to suppress HCV RNA level and to enhance the suppression of HCV RC by H_2O_2 (Fig. 4). The data suggest that endogenous generation of oxidants, either due to normal cell metabolism or triggered by HCV proteins expression [15, 16, 18-21], might be sufficient to modulate HCV RC in these cells. Most of all, however, the data indicate that ROS might suppress HCV RC while antioxidants, favor HCV RNA replication by antagonizing the suppressive effects of oxidants. This is the first study to demonstrate a modulation of viral replication machinery, independent of transcription factor modulation or oxidative damage to the virus particles, through redox signaling.

Nevertheless, the subgenomic and genomic replicons employed in this study do not generate virus particles and, therefore, while ROS clearly suppressed the activity of HCV RC in this study, the effects of ROS on the infectivity and morphogenesis of HCV remain to be determined. In this regard, it should be noted that some studies have correlated antioxidant therapy with a decreased viral titer, suggesting potential pro-viral effects of oxidants [59, 60]. Although these findings are difficult to reconcile, some of the discrepancies may be explained by the effects that some HCV proteins have on the host redox status [15-21]. It is also possible that antioxidants have other effects, such as on the immune system, which leads to an overall reduction of HCV viral load *in vivo*. Furthermore, oxidants/antioxidants may have other effects on different steps of HCV life cycle. In fact, whether calcium, the concentration of which changes physiologically with signaling, binds to cellular proteins or various constituents of HCV RC to differentially regulate the RNA replication versus other steps of viral life cycle remains to

be examined. Further studies that characterize the effects of redox and calcium signaling on other steps of HCV lifecycle will help resolve the complex mode of virus-host interactions involving ROS and calcium signaling during acute and chronic hepatitis C.

In conclusion, both endogenous and exogenous H_2O_2 can modulate HCV RC through redox signaling. Our findings indicate the importance of calcium and redox homeostasis during HCV infection and support a novel function of ROS, generated during inflammation, in the modulation of viral replication complex through signaling. Currently, 170 million people are estimated to be infected with HCV, including 4 million in the U.S. Current anti-HCV therapy show limited efficacy and severe side-effects. Future studies, aimed at studying the biological and pathological relevance of increased oxidative stress, may lead to the discovery of novel cellular/viral targets for anti-HCV therapy.

Acknowledgments

The authors would like to thank Drs. Hongqiao Zhang, Honglei Liu, and Alessandra Rinna for discussions. We also thank Alicia Blancas and Ben Alderete (Guava Technologies, Inc.) for assistance with apoptosis and mitochondrial membrane potential measurements. IFNγ was obtained through Reference Reagent Program at National Institute of Allergy and Infectious Diseases (NIAID). This work was supported by grants from National Institutes of Health and funds from the University of California, Merced.

List of Abbreviations

BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester; BSO, *L*-buthionine *S,R*-sulfoximine; $[Ca^{2+}]_i$, intracellular calcium concentration; DMEM, Dulbecco's modified essential medium; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic acid; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphodehydrogenase; GO, glucose oxidase; GSH, glutathione; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFNγ, interferon gamma; IP_3, inositol 1,4,5-triphosphate; IRES, internal ribosomal entry site; KRPH, Krebs-Ringer phosphate buffer; NAC, *N*-acetylcysteine; NFκB, nuclear factor kappa B; PBS, phosphate buffered saline; RC, replication complexes; ROS, reactive oxygen species; TG, thapsigargin

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Figure legends

Fig. 1. Suppression of HCV RNA by IFNγ and H_2O_2. SgPC2 cells were treated with various concentrations of H_2O_2 or IFNγ, as indicated. After 24 hrs, total RNA was analyzed for HCV RNA and GAPDH mRNA by northern blots, and the images were analyzed with a phosphoimager (Cyclone, Perkin-Elmer). The intensities of HCV RNA bands were normalized against GAPDH mRNA and expressed as the percentage of respective controls. Note that these and all other experiments in the subsequent figures were repeated 2 – 6 times, and data are presented as mean ± SEM of several independent experiments.

Fig. 2. Buffering $[Ca^{2+}]_i$ prevented the suppression of subgenomic and genomic HCV RC by ROS. SgPC2 cells were pretreated with 2 μM BAPTA-AM (Molecular Probes, Inc.) or the vehicle control alone (0.02 % dimethyl sulfoxide, DMSO) for 0.5 to 1 hr. (A) $[Ca^{2+}]_i$ was monitored after loading cells with Indo-1AM. H_2O_2 (100 μM) was added at 60 seconds. (B) SgPC2 cells were labeled with ^{3}H-uridine while treating them with H_2O_2 for 6 hrs in the presence of actinomycin D. Then, the RNA was isolated and analyzed, as described in Experimental Procedures. The gel was stained with ethidium bromide to compare the amount of rRNA present in each lane (the lower panel), which served as the loading control. (C) SgPC2 cells were treated with H_2O_2 for 30 min or 3 hrs, and the cytoplasmic extracts were subjected to in vitro replication assay. The RNA products were isolated and analyzed on a 1 % RNA gel. The gel was stained with ethidium bromide to compare the amount of rRNA in each lane (the lower panel) as a loading control. (D) SgPC2 cells were treated with H_2O_2 for 24 hrs. Then, total RNA was analyzed for HCV RNA and GAPDH mRNA by northern blots, and the images were analyzed

with a phosphoimager. The intensities of HCV RNA bands were normalized against GAPDH mRNA and expressed as the percentage of respective controls. H_2O_2, 36.8 ± 16.6 % of untreated control; H_2O_2 + BAPTA, 139.8 ± 20.2 % of BAPTA-treated control. (E) BAPTA removed the suppression of genomic HCV RNA replication by H_2O_2. Huh7 cells were transiently transfected with the genomic HCV RNA and then treated with H_2O_2 for 24 hrs, with 1 hr pretreatment with 2 μM BAPTA-AM or control DMSO. Total RNA was analyzed for HCV RNA by northern blot. GAPDH mRNA was also analyzed as the control. H_2O_2, 34.0 ± 4.0 % of untreated control; H_2O_2 + BAPTA, 111.5 ± 4.5 % of BAPTA-treated control.

Fig. 3. Continuous exposure to H_2O_2 suppressed HCV RNA replication. SgPC2 cells were treated with GO for 24hrs, with and without co-treatment with 130 - 200 U/ml of catalase or with 1 hr pretreatment with 2 μM BAPTA-AM or DMSO. (A-B) HCV RNA and GAPDH mRNA levels were determined by northern blot analysis and the images were analyzed with a phosphoimager. The intensities of HCV RNA bands were normalized against GAPDH mRNA and expressed as the percentage of control. Data represent mean ± SEM. (C) HCV NS5A protein level was analyzed by western blot, as described in Experimental Procedures.

Fig. 4. GSH and HCV RNA replication. SgPC2 cells were incubated with 20 - 40 μM BSO with and without H_2O_2 for 24 hrs and analyzed for GSH (A) or HCV RNA and GAPDH mRNA levels by northern blots (B). Total GSH was expressed as nmol/mg total protein. The RNA bands were analyzed with a phosphoimager. GAPDH mRNA served as the control. Data represent mean ± SEM. Star (*) indicates statistically significant difference by Student's T-test ($P \leq 0.05$).

Fig. 5. H_2O_2 induced calcium release from the ER. SgPC2 cells were loaded with indo-1 AM, and $[Ca^{2+}]_i$ was monitored in the absence of extracellular calcium, unless specified otherwise. (A) H_2O_2 or water was added at 60 sec and calcium was monitored in the presence and absence of 1.3 mM extracellular calcium. (B-C) Cells were pretreated with (B) H_2O_2 for 13 min or (C) 400 nM TG or control DMSO for 10 min prior to calcium measurement. Arrows indicate the addition of peroxide (A and C) or TG (B).

Fig. 6. The suppression of HCV replication by H_2O_2 required internal calcium store(s). SgPC2 cells were incubated in normal medium (10 % FBS), 0 % FBS, serum-starved overnight with 0.5 % FBS and then incubated in 0% FBS for 1 hr, in KRPH, or pretreated with DMSO control or 5 μM ionomycin in calcium-free KRPH buffer for 15 min. Then, the cells were treated with H_2O_2 for 30 min. The cytoplasmic lysates were prepared and analyzed for *in vitro* HCV replication. rRNA panel shows RNA loading. HCV RNA bands were analyzed with a phosphoimager, normalized against the rRNA bands, and expressed as the percentages of respective controls. Data represent mean ± SEM.

Fig. 7. Elevated calcium might be sufficient to suppress HCV replication. (A) SgPC2 cells were treated with 400 nM and 2 μM TG, DMSO control, H_2O_2, or 10 μM ionomycin for 15 min. Then, cytoplasmic lysates were prepared and analyzed for *in vitro* HCV replication. Data represent mean ± SEM. (B) $CaCl_2$ was added to untreated cytoplasmic lysates at concentrations shown prior to *in vitro* replication assay. rRNA panel shows RNA loading. The RNA bands

were analyzed with a phosphoimager, normalized against the rRNA bands and expressed as the percentage of respective controls. Data represent mean ± SEM. (C) $CaCl_2$ was added to purified full-length NS5B at concentrations shown prior to *in vitro* RdRp assay. $ZnCl_2$ (0.5mM) was used as a positive control.

Figure 1

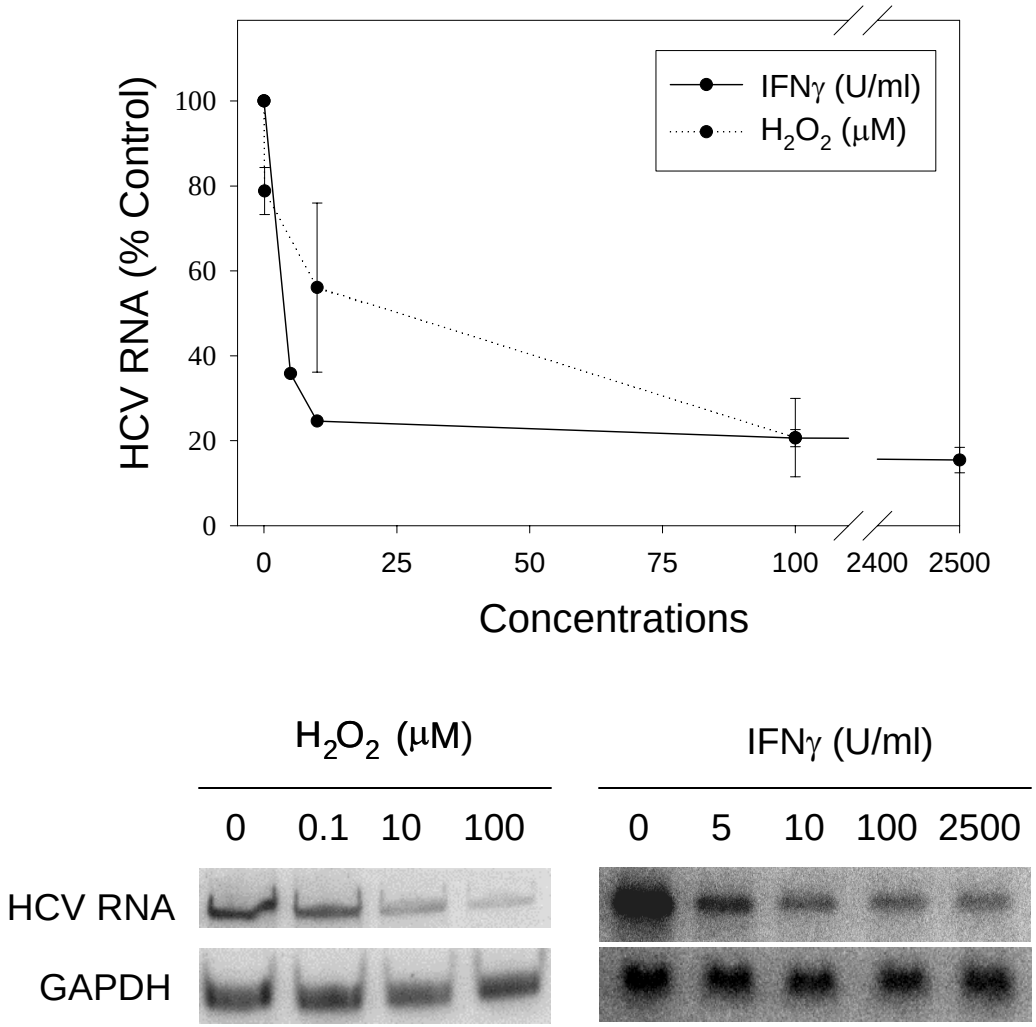
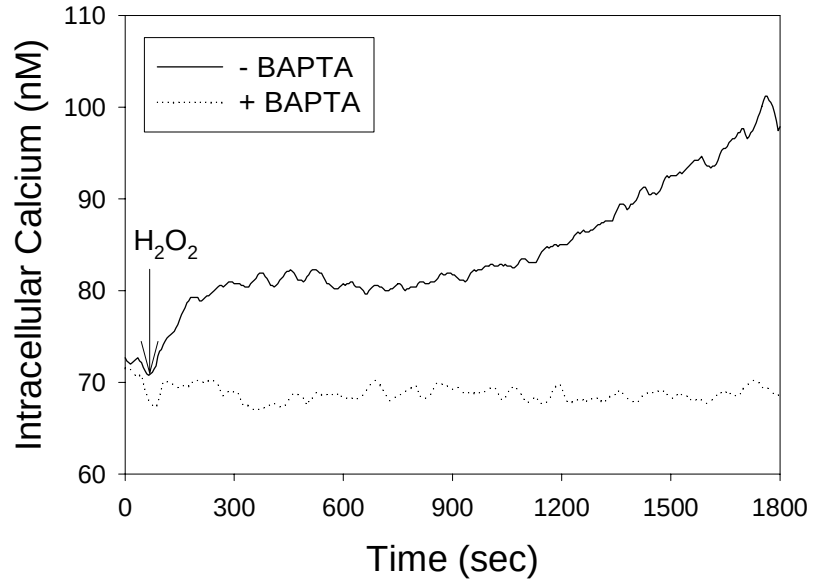

IFNγ (U/ml)
H2O2 (μM)
HCV RNA (% Control)
100
80
60
40
20
0
0
25
50
75
100
2400
2500
Concentrations
H2O2 (μM)
0
0.1
10
100
IFNγ (U/ml)
0
5
10
100
2500
HCV RNA
GAPDH

Figure 2

(A)


110
100
90
80
70
60
0
300
600
900
1200
1500
1800
Intracellular Calcium (nM)
Time (sec)
- BAPTA
+ BAPTA
H2O2

(B)

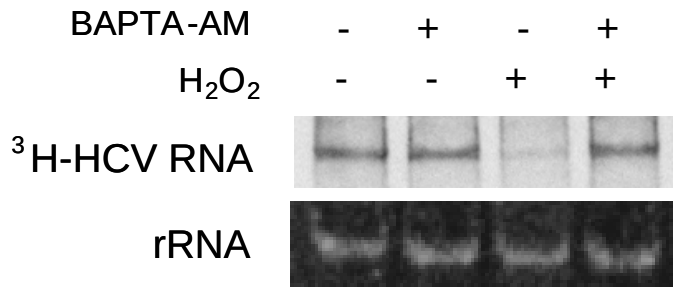

BAPTA-AM - + - +
H2O2 - - + +
3H-HCV RNA
rRNA

Figure 2

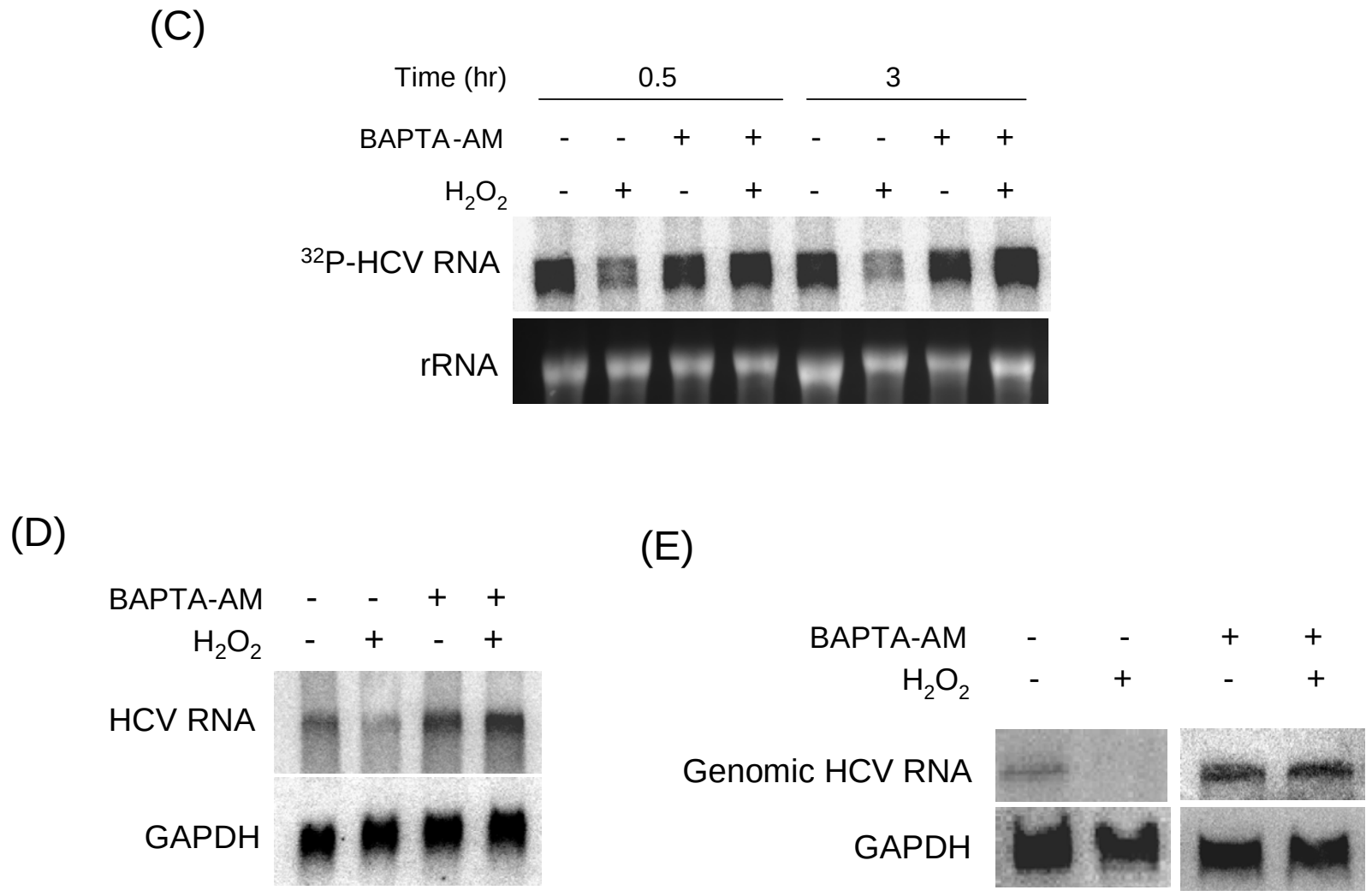
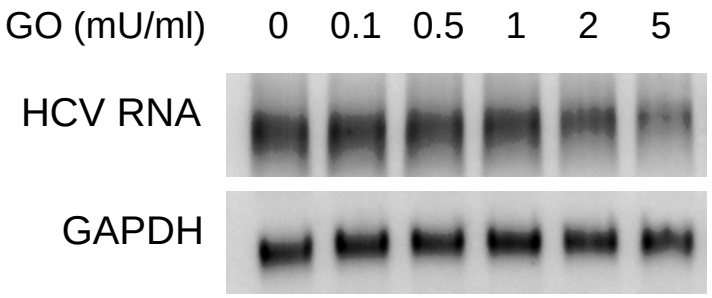

(C)
Time (hr)
0.5
3
BAPTA-AM
- - + + - - + +
H2O2
- + - + - + - +
32P-HCV RNA
rRNA
(D)
BAPTA-AM
- - + +
H2O2
- + - +
HCV RNA
GAPDH
(E)
BAPTA-AM
- - + +
H2O2
- + - +
Genomic HCV RNA
GAPDH

Figure 3

(A)


GO (mU/ml)
0
0.1
0.5
1
2
5
HCV RNA
GAPDH

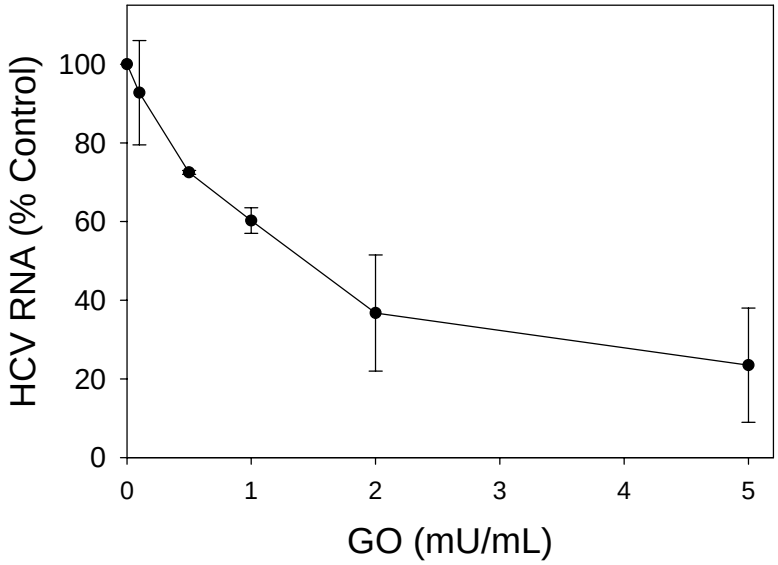
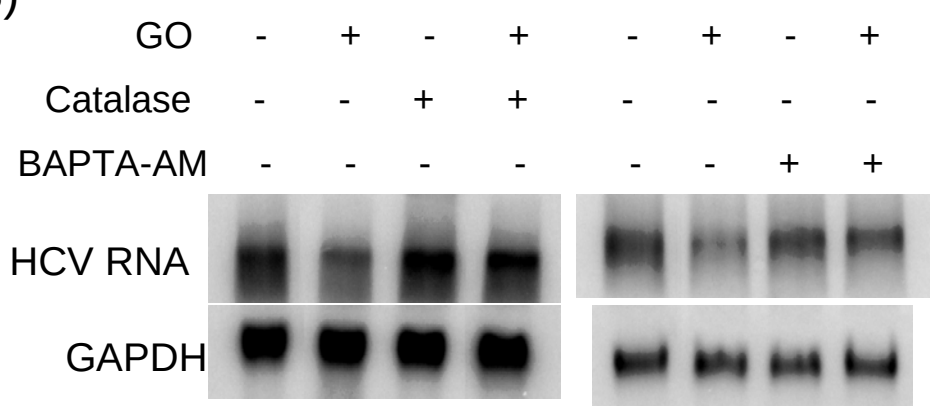
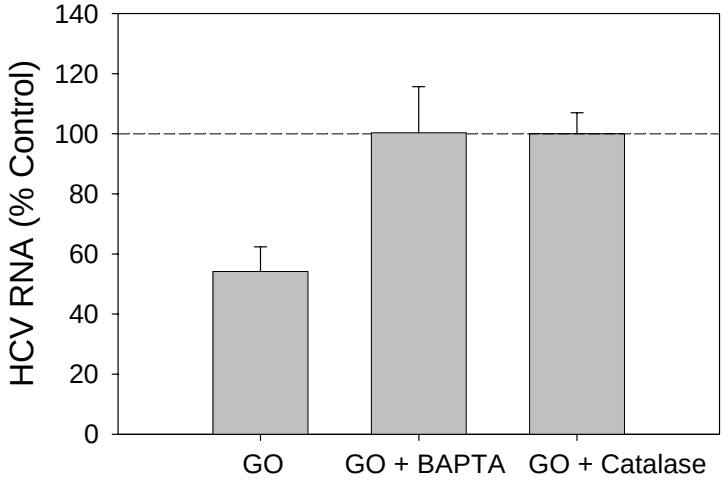

HCV RNA (% Control)
100
80
60
40
20
0
0
1
2
3
4
5
GO (mU/mL)

Figure 3

(B)


GO - + - + - + - +
Catalase - - + + - - - -
BAPTA-AM - - - - - - + +
HCV RNA
GAPDH


HCV RNA (% Control)
140
120
100
80
60
40
20
0
GO
GO + BAPTA
GO + Catalase

(C)

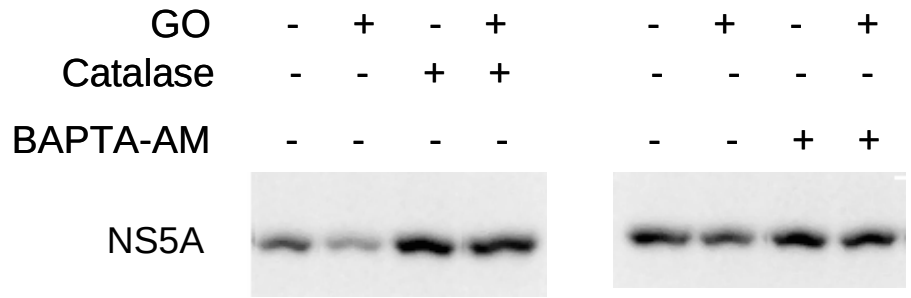
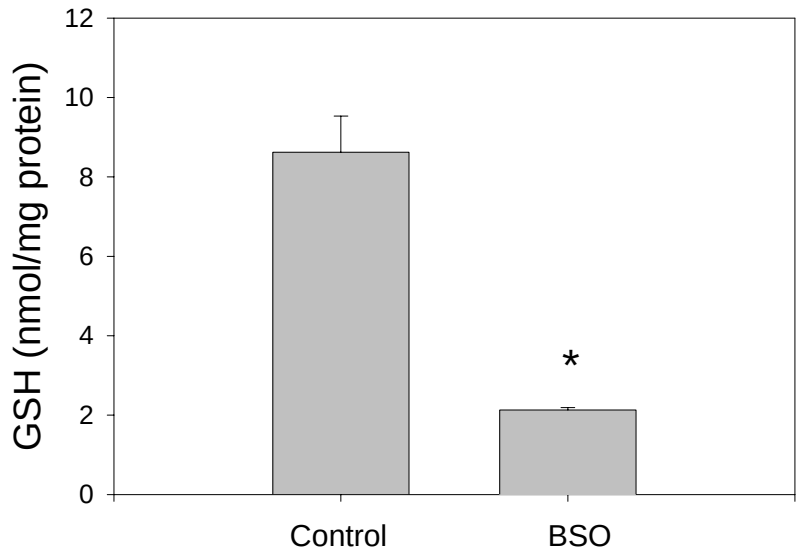
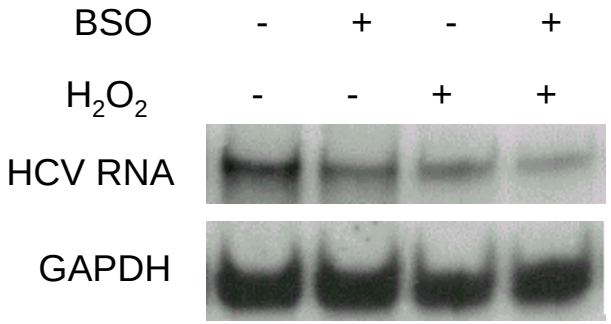

GO - + - + - + - +
Catalase - - + + - - - -
BAPTA-AM - - - - - - + +
NS5A

Figure 4

(A)


GSH (nmol/mg protein)
12
10
8
6
4
2
0
*
Control
BSO

(B)


BSO - + - +
H2O2 - - + +
HCV RNA
GAPDH

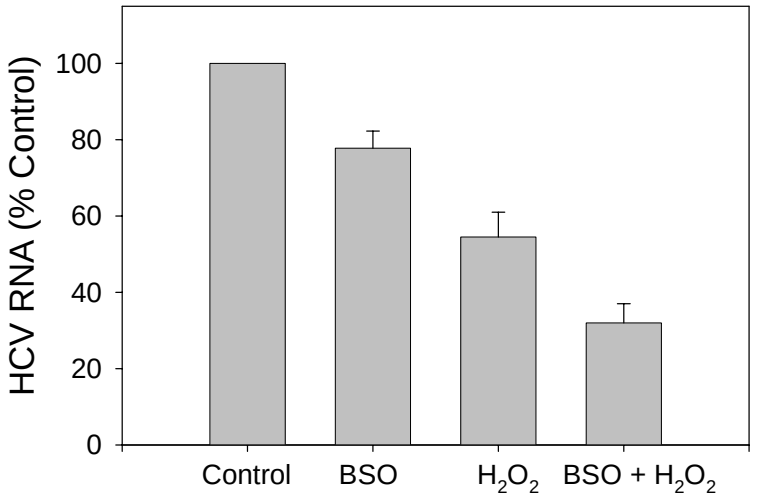
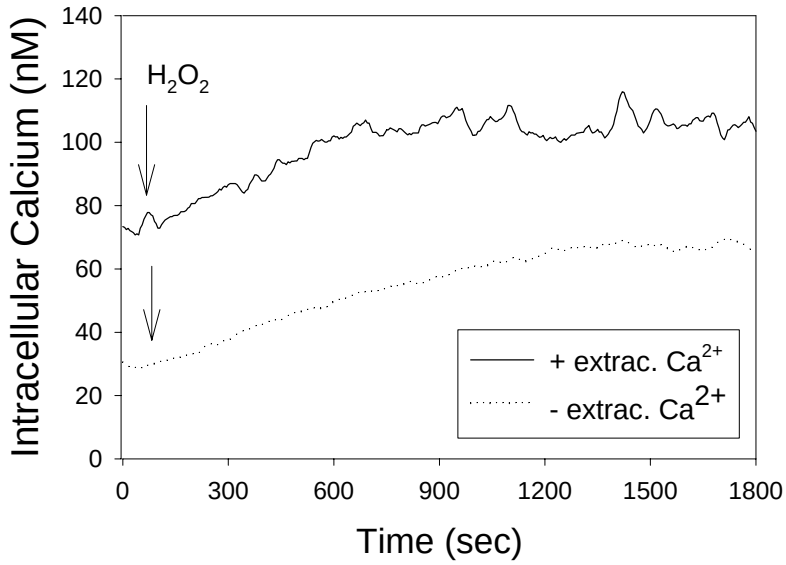

HCV RNA (% Control)
100
80
60
40
20
0
Control
BSO
H2O2
BSO + H2O2

Figure 5

(A)


Intracellular Calcium (nM)
140
120
100
80
60
40
20
0
H_2O_2
0
300
600
900
1200
1500
1800
Time (sec)
+ extrac. Ca^{2+}
- extrac. Ca^{2+}

(B)

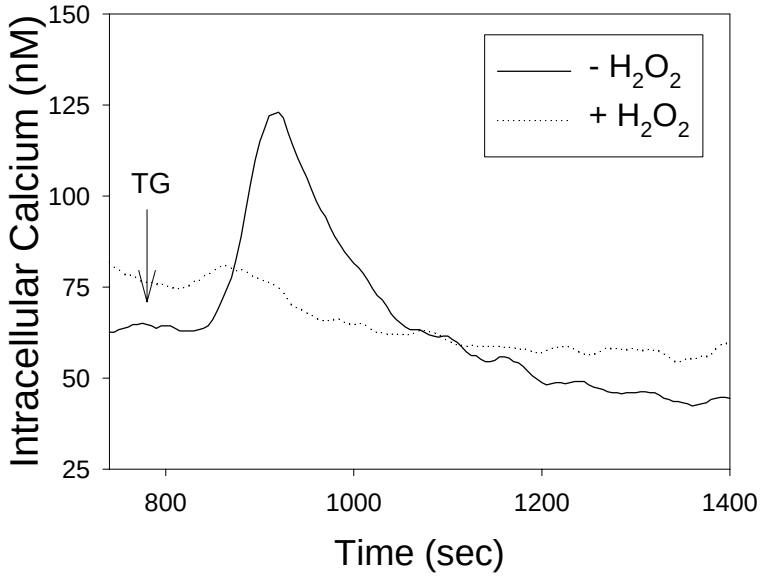

Intracellular Calcium (nM)
150
125
100
75
50
25
TG
800
1000
1200
1400
Time (sec)
- H_2O_2
+ H_2O_2

Figure 5

(C)

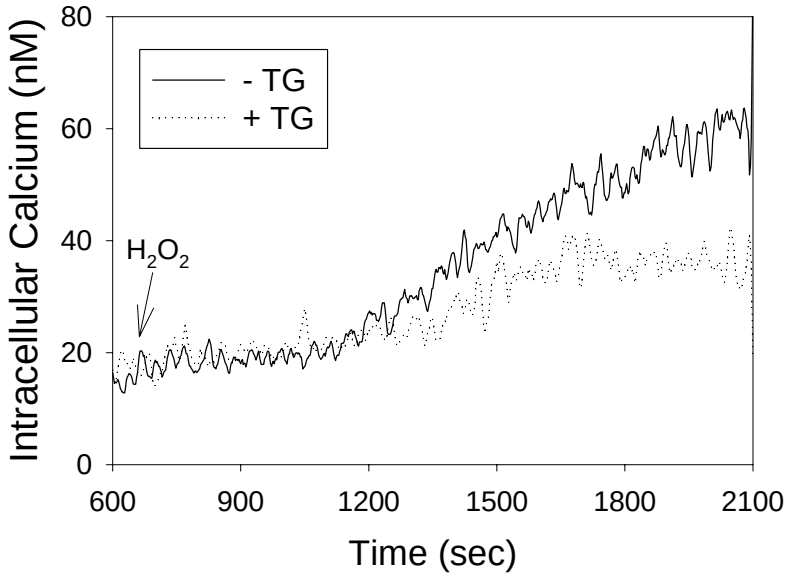

Intracellular Calcium (nM)
80
60
40
20
0
- TG
+ TG
H_2O_2
600
900
1200
1500
1800
2100
Time (sec)

Figure 6

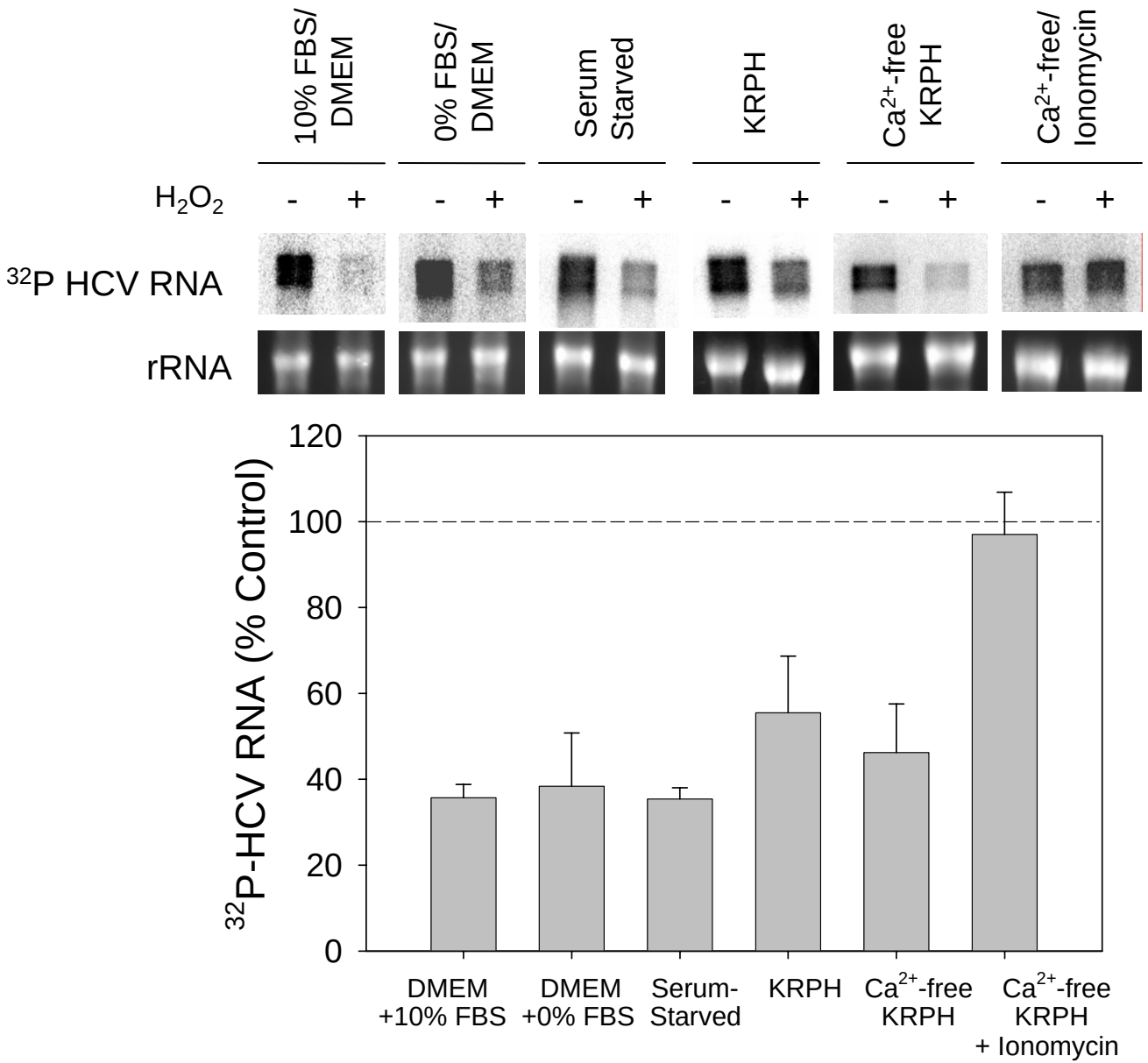
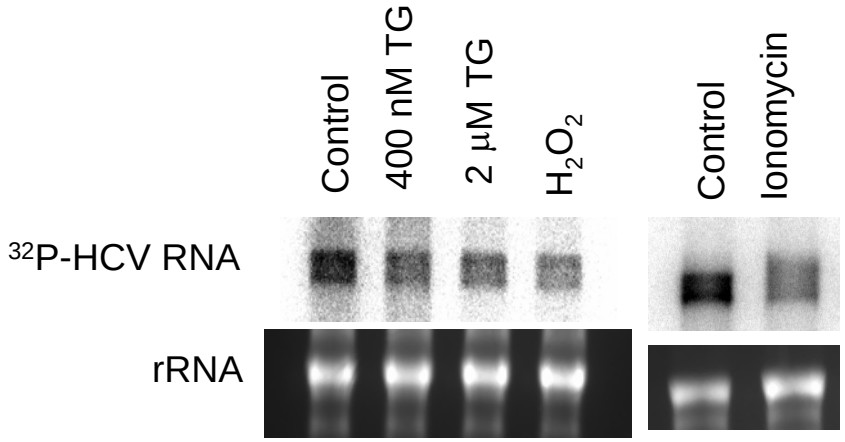

10% FBS/ DMEM
0% FBS/ DMEM
Serum Starved
KRPH
Ca2+-free KRPH
Ca2+-free/ Ionomycin
H2O2
- + - + - + - + - + - +
32P HCV RNA
rRNA
32P-HCV RNA (% Control)
120
100
80
60
40
20
0
DMEM +10% FBS
DMEM +0% FBS
Serum-Starved
KRPH
Ca2+-free KRPH
Ca2+-free KRPH + Ionomycin

Figure 7

(A)


Control
400 nM TG
2 μM TG
H_2O_2
Control
Ionomycin
^{32}P-HCV RNA
rRNA

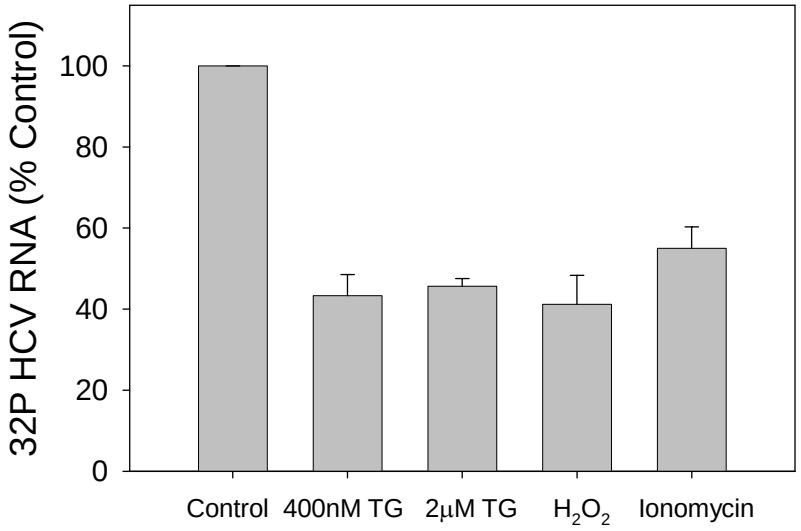
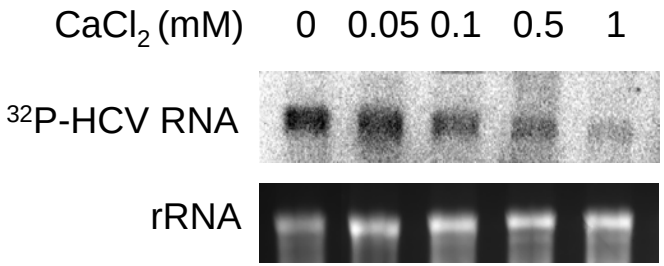
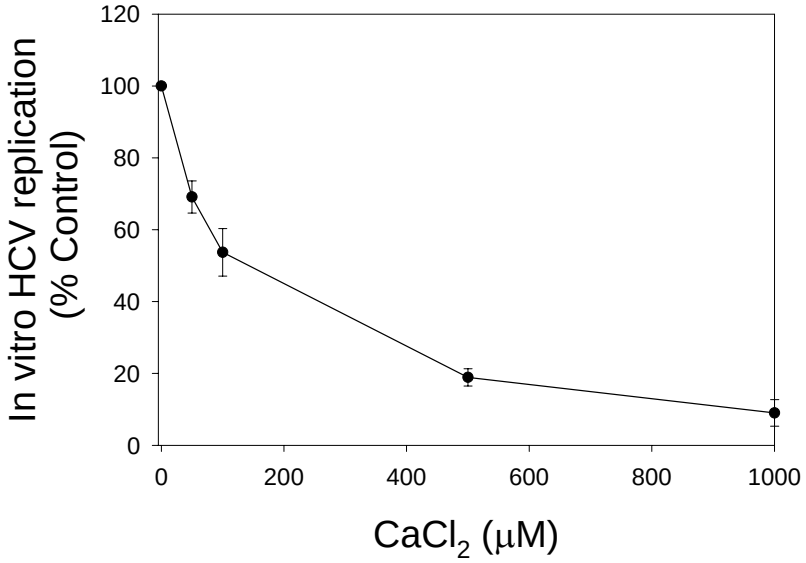

32P HCV RNA (% Control)
100
80
60
40
20
0
Control
400nM TG
2μM TG
H_2O_2
Ionomycin

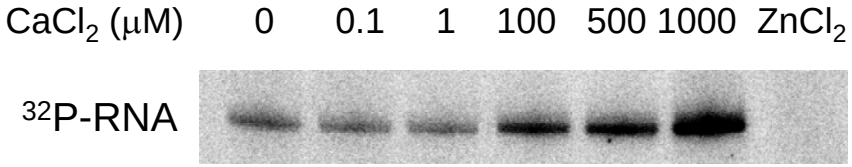
Figure 7

(B)


CaCl2 (mM) 0 0.05 0.1 0.5 1
32P-HCV RNA
rRNA


In vitro HCV replication (% Control)
120
100
80
60
40
20
0
0
200
400
600
800
1000
CaCl2 (μM)

(C)


CaCl2 (μM) 0 0.1 1 100 500 1000 ZnCl2
32P-RNA