

HEPATITIS C VIRUS CORE PROTEIN INHIBITS MITOCHONDRIAL ELECTRON TRANSPORT AND INCREASES ROS PRODUCTION

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Running title: HCV core inhibits mitochondrial complex I

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Hepatitis C infection causes a state of chronic oxidative stress which may contribute to fibrosis and carcinogenesis in the liver. Previous studies have shown that expression of the HCV core protein in hepatoma cells depolarized mitochondria and increased reactive oxygen species (ROS) production, but the mechanisms of these effects are unknown. In this study we examined the properties of liver mitochondria from transgenic mice expressing HCV core protein, and from normal liver mitochondria incubated with recombinant core protein. Liver mitochondria from transgenic mice expressing the HCV proteins core, E1 and E2 demonstrated oxidation of the glutathione pool and a decrease in NADPH content. In addition, there was reduced activity of electron transport complex I, and increased ROS production from complex I substrates. There were no abnormalities observed in complex II or complex III function. Incubation of control mitochondria in vitro with recombinant core protein also caused glutathione oxidation, selective complex I inhibition, and increased ROS production. Proteinase K digestion of either transgenic mitochondria or control mitochondria incubated with core protein showed that core protein associates strongly with mitochondria, remains associated with the outer membrane, and is not taken up across the outer membrane. Core protein also increased Ca²⁺ uptake into

isolated mitochondria. These results suggest that interaction of core protein with mitochondria and subsequent oxidation of the glutathione pool and complex I inhibition may be an important cause of the oxidative stress seen in chronic hepatitis C.

Hepatitis C virus (HCV) infection produces acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1). Severity and rate of progression of the disease are highly variable and may reflect both host and viral factors (2) but the mechanisms of pathogenesis are incompletely understood. Since current anti-viral treatment can only eliminate the virus in about 50% of patients (3-5), therapies to reduce disease progression in chronically infected individuals would be of great benefit. Thus, understanding the mechanisms of HCV pathogenesis is an important goal of HCV research. Numerous studies have shown that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases (6;7) and a prospective study showed improvement in liver injury in chronic hepatitis C with anti-oxidant treatment (8). Previous studies from our lab and others have shown that HCV core protein induces the production of reactive oxygen species (ROS) (9-12) but the mechanism of the core-associated increase in ROS production is not understood.

HCV core protein localizes to ER (13;14), fat droplets (15;16) and nucleus (17) as well as

mitochondria (9;18;19). It has been shown to produce multiple cellular effects including changes in gene transcription, signal transduction, immune presentation, cell cycle regulation and apoptosis (20-25). Despite this evidence, it is not known whether HCV core protein has a direct functional effect on mitochondria and whether this accounts for its ability to increase ROS. To clarify these questions, we investigated the interaction of HCV core protein with mitochondria in transgenic mice and by direct interaction of recombinant core protein with isolated mitochondria.

HCV protein expression caused an increase in mitochondrial ROS production, an oxidation of the mitochondrial glutathione pool, inhibition of electron transport and an increase in ROS production by mitochondrial electron transport complex I. Direct incubation of isolated mitochondria with HCV core protein resulted in an increase of Ca^{2+} influx and ROS production and reproduced glutathione oxidation and the reduction in complex I function. These results suggest that direct interaction of core protein with mitochondria is an important cause of the oxidative stress seen in chronic hepatitis C.

MATERIALS AND METHODS

Generation of transgenic mice. The transgene, pAlbSVPA-HCV-S, containing the structural genes (core, E1, E2, and p7, nucleotides 342-2771) of hepatitis C virus genotype 1b, strain N, under the control of the murine albumin promoter/enhancer was described in detail by Lerat et al (26). This construct was injected into 1-cell F₂ zygotes of C57BL/6J x C3H/HeJ mice. Fourteen transgene-positive pups were obtained, out of 121 live births, as screened by polymerase-chain reaction, using two pairs of primers spanning the promoter to the core gene for one pair and the E2 gene for the other pair. Positive results were confirmed by Southern blot analysis. Each of these transgenic founder mice were backcrossed to C57BL/6J. One transgenic line, designated SL-139 was selected for subsequent experiments. Female HCV transgenic mice were used at 5-7 months of age and age matched non transgenic littermates were used as controls. All animal procedures were performed according to the National Institutes of Health Guidelines and

were approved by the Institutional Animal Care and Use Committee.

RNA extraction and RT-PCR. Liver samples were collected from transgenic mice of the first backcross (N₁) generation, following carbon dioxide euthanasia. RNA was extracted from the liver using Trizol (Sigma, St Louis MO). Contaminating DNA was removed by brief treatment with DNase, which was removed by phenol/chloroform extraction. Reverse transcription was performed using Omniscript (Qiagen, Valencia CA), followed by PCR amplification of the E2 region using RedTaq (Sigma) according to manufacturers' protocols. The PCR products were analyzed by electrophoresis on 2% agarose gels.

Determination of intrahepatic core protein concentration. SL-139 mice were sacrificed by CO₂ asphyxiation, and liver protein was extracted in cold RPMI-1640. Quantity of HCV core protein was determined with an HCV core ELISA kit (trak-C™, Ortho Clinical Diagnostics, Raritan, NJ). Briefly, samples were mixed with a pretreatment buffer containing detergents and incubated at 56°C for 30 min. A set of 6 standards, supplied by the manufacturer, which contained 100, 50, 15, 5, 1.5, and 0 pg/ml of HCV core antigen, was prepared. Standards, pretreated samples and controls were transferred to a micro-well plate coated with capturing monoclonal antibodies against HCV core protein, and incubated at 25°C for 60 min. After washing, monoclonal antibody F(ab')₂ fragments conjugated to horseradish peroxidase were added and incubated at 25°C for 30 min. After a final wash step, wells were incubated in the dark with substrate for 30 min. To stop color development, sulfuric acid was added and absorbance was measured with a micro-well plate reader at a wavelength of 490 nm with a reference wavelength of 620 nm. The concentration of HCV core antigen in each sample was determined from the standard curve.

Isolation of mitochondria. Liver mitochondria were isolated by a modification of the method of Johnson and Hardy (27-29). In brief, liver (400 mg) was minced on ice and transferred (10% W/V) to isolation buffer (250 mM Sucrose, 10 mM HEPES, 0.5 mM EGTA, 0.1% BSA, pH 7.4). The sample was gently homogenized by 3-4

strokes with a Dounce homogenizer and loose-fitting pestle. The homogenate was centrifuged at 500 g for 5 min at 4°C. The supernatant fraction was retained, whereas the pellet was washed with isolation buffer and centrifuged again. The combined supernatant fractions were centrifuged at 7800 g for 10 minutes at 4°C to obtain a crude mitochondria pellet. The mitochondria pellet was resuspended in isolation buffer (without EGTA and BSA) and centrifuged again at 7800g for 10 min. An aliquot was removed for determination of protein concentration by the Bio-Rad assay kit, using bovine serum albumin as the standard.

Determination of glutathione content. Liver tissue samples (50-75 mg) and mitochondrial samples (2 mg) were sonicated using a Branson Sonifer 450 (VWR Scientific Products, West Chester PA) for 15s at power setting 3 in ice-cold 5% trichloroacetic acid and centrifuged at 3,000 g at 4°C for 10 min. The concentration of reduced GSH was measured by the thioester method using the GSH-400 kit (Oxis International Inc., Portland, Oregon). Total glutathione content of samples was measured by the glutathione reductase-DTNB recycling assay (30) using a commercial kit (GSH-412, Oxis International).

To measure the effect of recombinant core protein (amino acids 1-179, kindly provided by S. Watowich) on mitochondrial glutathione, freshly isolated mitochondria were suspended in PBS and incubated at 25°C for 5 min with or without core protein. Proteins were precipitated and thiols stabilized by subsequent addition of sulfosalicylic acid to a final concentration of 5%. To confirm that decreases in reduced GSH measured by the thioester method were indeed a result of oxidation, parallel mitochondrial samples were either further oxidized by exposure to 0.2 mM tBOOH for 5 min, or reduced by freeze thaw followed by incubation with glutathione reductase (4.1 units/ml) and NADPH (1 mM) for 5 min at 25°C. Following reduction, samples were precipitated with sulfosalicylic acid and processed as described. Control experiments showed that this tBOOH treatment fully oxidized the glutathione pool under these conditions and it was used to determine the background value for the assay.

NADPH and glutathione reductase measurement. NADPH was measured in isolated

mouse liver mitochondria by the method described by Zhang et al. (31). Mitochondrial pellets were suspended in 0.1 M Tris, 10 mM EDTA, 1% Triton X-100, pH 7.6, and then centrifuged at 20,000 x g for 10 min to remove membrane debris and obtain clear supernatant. Absorbance at 340 nm was determined in untreated supernatants (A_1), and after specific oxidation of NADPH to NADP⁺ with glutathione reductase and GSSG (A_2). A_1 - A_2 represented the amount of NADPH in the sample (31). Glutathione reductase (GR) activity was measured as the rate of decrease in absorbance at 340 nm caused by the oxidation of NADPH (GR assay kit, Sigma, St. Louis, MO). A reaction with assay buffer instead of mitochondrial sample was run as a blank.

Measurement of oxygen consumption. Oxygen consumption of isolated mitochondria was measured at 25°C using a model 782 oxygen meter system and model 1302 Microrcathode oxygen electrode (Strathkelvin, Glasgow UK). Mitochondrial pellet (1-1.5 mg/ml) was added to the 1 ml sample chamber filled with respiration buffer (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5mM KH₂PO₄, 0.05 mM EDTA and 5mM HEPES, pH 7.4) and allowed to equilibrate with magnetic stirring. Complex I-supported state 4 respiration was initiated by addition of 5 mM glutamate and 5 mM malate to the sample chamber. Subsequent addition of 100 nmole ADP initiated complex I –supported state 3 respiration. After returning to state 4 respiration, maximum oxygen consumption (uncoupled respiration) was measured by adding 5 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). Similarly, complex II-supported state 3 and 4 respiration was measured using 5 mM succinate.

Effects of tBOOH and HCV core protein on mitochondrial respiration. Isolated hepatic mitochondria were incubated with 100 μM tBOOH and/or 1, 10 or 100 ng of recombinant HCV core protein per mg mitochondrial protein at 25°C for 5 min. Aliquots of the mitochondrial suspension were added to the sample chamber for analysis of rates of oxygen consumption. P:O ratio and FCCP induced consumption rates were calculated as described by Estabrook (32).

Measurement of Complex I and III activity.

Enzyme activity assays were performed at 25°C by previously established methods (33;34). Submitochondrial particles were prepared from mitochondria by incubation for 3min at 37°C followed by sonication in a microcentrifuge tube immersed in ice water. Submitochondrial particles were pelleted at 15,000 g for 10 min and 50 µg were used for each assay. In some instances SMPs were reduced by incubation with dithiothreitol (100 µM) for 10 min at 0°C. Complex I activity (NADH-decylubiquinone oxidoreductase) was measured as the initial (5 min) rate of decrease of A_{340} using the acceptor 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone (DB 80 µM) and 200 µM NADH as donor in 10mM Tris (pH 8.0) buffer containing 1mg/ml BSA, 0.24 mM KCN and 0.4 µM antimycin A. Complex III activity (ubiquinol cytochrome c reductase) was measured at 550nm using 40 µM oxidized cytochrome c as the acceptor and 80 µM decylubiquinol as the donor in 10 mM KH_2PO_4 (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 µM rotenone, 0.2 mM ATP for 2min. The addition of 1 µM antimycin A allowed us to distinguish between the reduction of cytochrome c catalyzed by complex III and the nonezymatic reduction of cytochrome c by the reduced quinone. Extinction coefficients were 6200 L/mol cm for NADH and 2.11×10^4 L/mol cm for oxidized cytochrome c.

Measurement of ROS production in mitochondria.

Mitochondrial ROS production was determined with the oxidation sensitive fluorogenic precursor dihydrodichlorocarboxyfluorescein diacetate (DCFDA, Molecular Probes) (35). Briefly, each well of a 96-well microtiter plate was filled with respiration buffer containing 1 µM of DCFDA and 0.5 mg/ml of mitochondrial particles (final volume 0.2 ml). The reaction was started by addition of 5 mM glutamate or 5 mM succinate and then incubated at 30°C in a shaker for 30-60 min. Fluorescence was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA) at excitation of 485 nm and emission of 530 nm. Some experiments included inhibitors, 5 µM FCCP, 1 µM rotenone, or 10 µM BAPTA-AM (Molecular Probes). In some experiments mitochondria from control mice

were incubated for 5 min with HCV core protein. For Ca^{2+} induced ROS production, the mitochondrial suspension was first exposed to 125 µM Ca^{2+} for 30min on ice.

Measurement of mitochondrial Ca^{2+} . For Ca^{2+} determination, mitochondria (0.5 mg/ml) were incubated for 1 h at 4 °C with the mitochondrial Ca^{2+} indicator Rhod-2 AM (4 µM, Molecular Probes), washed twice in 0.25 M sucrose, 2 mM K-Hepes buffer, and diluted to a final concentration of 0.33 mg protein/ml in 100 mM KCl, 20 mM Tris, 20 mM Hepes, 10 mM NaCl, 5 mM Na-succinate, 1 mM KH_2PO_4 , 20 µM K-EGTA, 2 µM rotenone, and 1 µg/ml oligomycin, pH 7.2. The mitochondria were exposed to further treatment with or without exogenous HCV core protein 10 ng/mg protein on ice for 5 min, and FCCP (5 µM) on ice for 30 min as indicated. Mitochondrial suspensions were subsequently exposed to 125 µM Ca^{2+} containing respiration buffer for 30 min on ice. The red fluorescence of Rhod-2 was measured in 96 -well plates in a CytoFluorII fluorescence plate reader at excitation 530 nm and emission 590 nm.

Western blotting. Samples were lysed in 625 mM Tris, pH 7.4, 2% sodium dodecyl sulfate (SDS), 1 mM EDTA and 1% protease inhibitor cocktail (Sigma). Mitochondria and liver lysates were centrifuged at 7,900g for 10 minutes and the supernatants (30 µg protein) were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio Rad), blocked overnight at 4°C with 5% nonfat dried milk, 0.1% Tween 20 in phosphate-buffered saline (PBS), and subsequently incubated for 2 h at room temperature with mouse monoclonal antibody to human hepatitis C virus core protein (1:450, Anogen, Mississauga, ON), rabbit polyclonal anti-Tom20 antibody (1:2000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-cytochrome C antibody (1:2000, R & D System, Inc., Minneapolis, MN), mouse monoclonal IgG anti Complex III core 2 subunit antibody (1:5000, Affinity Molecular probes Inc) or mouse monoclonal anti-mitochondrial Heat Shock Protein 70 antibody (1:2,000, affinity BioReagents, Golden, Co). The membranes were

washed, incubated with appropriate secondary antibodies, and detected with the ECLplus chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Assessment of core protein localization by proteolysis. Isolated mitochondria from transgenic liver were incubated in respiration buffer with proteinase K (50 $\mu\text{g/ml}$) for 30 min at 4° C. After incubation, protease activity was inhibited by addition of PMSF to a final concentration of 2 μM , followed by incubating on ice for an additional 10 minutes. Then 10 μg of the mitochondrial suspension was subjected to Western blotting without centrifugation. 1% Triton X-100 was used in some experiments to disrupt the mitochondrial membranes.

Statistics. Results are expressed as mean \pm SE. Student's *t* test was used for statistical analyses. $P < 0.05$ was considered significant.

RESULTS

Characteristics of HCV Transgenic Mice.

We developed a new transgenic mouse model in order to study the effects of viral proteins on native liver mitochondria. Lerat et al (26) reported the occurrence of steatosis and hepatocellular carcinogenesis in a transgenic line S-N/863 encoding the structural genes of HCV. Transgene RNA expression in the liver of this lineage is detectable by RT-PCR, and Northern blot analysis, but protein expression was not detectable by immunoblotting and thus is lower than that typically seen in patients with chronic hepatitis C (36). Using the same construct (Fig. 1A), we derived five transgenic lines that expressed HCV RNA, as demonstrated by RT-PCR, three of which expressed the HCV core protein detectable by immunoblotting. Northern blot analysis shows that these mouse lines produced two HCV-specific RNA transcripts, 3.4 kb and 4.2 kb (Fig. 1B). The spleen was the only other organ which was positive by RT-PCR, at a relatively reduced level (data not shown). One of the three lines, designated as SL-139 was backcrossed to C57BL/6J and the offspring were used in all experiments reported here. Core protein concentration determined by ELISA in liver

extracts was 0.59 ± 0.23 pg core/ μg liver protein (mean \pm SD).

Presence of core protein in transgenic mitochondria. To test for expression of HCV core protein in the mitochondria, western blot analysis was performed on crude mitochondria from transgenic and control livers (Fig. 2A). As previously reported, HCV core protein was present in the mitochondrial pellet (9;18) and two forms of HCV core protein (p23 and p21) (13;14;17;37;38) were detected. Core protein relative abundance was greater in mitochondria than in whole liver homogenate (Fig. 2A) and comparison to recombinant core (amino acids 1-179) standards suggested that p21 is similar in size to core 1-179. Content of core protein was on the order of 2-5 ng core/mg mitochondrial protein (Fig. 2B).

Oxidant status of transgenic mitochondria.

To determine if SL-139 mice have altered oxidative status of their mitochondria we measured total hepatic and mitochondrial content of reduced (GSH) and total (GSH + GSSG) glutathione. There was no significant difference in whole liver total glutathione between control and transgenic mice (36.6 ± 1.9 vs. 32.7 ± 3.4 nmol/mg protein), approximately 97% of the whole liver glutathione pool was in the reduced form, and there was no effect of HCV transgene expression on the proportion of reduced vs. total glutathione (Fig. 3A,B). Liver mitochondrial total glutathione was 8.2 ± 1.2 nmole/mg protein in control animals and was not different in transgenic animals (Fig. 3C). However, mitochondrial reduced GSH content was significantly decreased in transgenics (Fig. 3D, $p < 0.01$) demonstrating a baseline oxidation of the mitochondrial glutathione pool in these animals.

To determine if capacity to maintain a reduced mitochondrial environment was altered we further measured mitochondrial glutathione reductase (GR) activity and NADPH. GR activity was similar in control vs. transgenic mitochondria (2.58 ± 0.27 vs. 2.49 ± 0.34 mU/mg mitochondrial protein, $n=8$). However, mitochondrial NADPH content was decreased in transgenic as compared to control mitochondria (0.80 ± 0.17 vs. 1.32 ± 0.17 nmoles/mg mitochondrial protein, $n=9$, $P < 0.05$.) Total (NADP⁺ + NADPH) content was

unchanged. This result further confirms the oxidized phenotype of the transgenic mitochondria.

Effects of transgene on mitochondrial respiration. Previous studies have demonstrated that core protein causes depolarization of the mitochondrial membrane potential ($\Delta\Psi$) (10). This depolarization could result either from decreased electron transport or increased proton leak. To distinguish between these possibilities, we measured O_2 consumption in the transgenic mouse liver mitochondria. The results are presented in Figure 4. Transgene expression significantly reduced the P:O ratio when the complex I substrates glutamate/malate were used (Fig. 4A, $P < 0.05$), but had no effect on P:O ratio when the complex II substrate, succinate, was used (Fig. 4C). Maximal O_2 consumption in the presence of FCCP was also reduced in transgenic mitochondria, but this change was not significant (Fig. 4B). These data suggest that inhibition of electron transport at complex I and not proton leak is the primary mechanism of reduced $\Delta\Psi$ after HCV core expression.

To confirm if HCV proteins also sensitized mitochondria to oxidative stress, we treated mitochondria with tBOOH. A 5-min exposure at 25°C to 100 μ M tBOOH had no effect on control mitochondria but it significantly reduced complex I mediated P:O ratio and maximal O_2 consumption rate in transgenic mitochondria (Fig. 4A,B). There were no changes in the same parameters measured when succinate was used as substrate (Fig. 4C,D).

In order to determine if the inhibition of O_2 consumption from complex I substrates was a direct result of complex inhibition, we measured complex I and complex III activities in submitochondrial particles (SMPs). Liver SMPs derived from transgenic mice had an approximately 25% reduction of complex I activity compared to that of SMPs from control liver (Fig. 5A, $P < 0.001$). Decreased activity could not be reversed by reduction of SMPs with dithiothreitol (DTT). However, complex III activity was normal (Fig. 5B). The magnitude and specificity of this effect was similar to that seen for oxygen consumption demonstrating that complex I inhibition was the primary cause of the reduction in respiration in transgenic mitochondria.

Effects of recombinant HCV core protein on isolated mitochondria. To assess whether the effects seen in the transgenic mitochondria result directly from the interaction of core protein with mitochondria we measured mitochondrial respiration and enzyme activity after incubation of normal liver mitochondria with purified HCV core protein. Mitochondria were incubated for 5 min at 25°C with varying concentrations of recombinant core protein. At concentrations of core protein present in the transgenic mitochondria (from 1-10 ng core protein/mg mitochondrial protein), there was a specific reduction of O_2 consumption and P:O ratio from glutamate/malate with no effect on oxidation of succinate (Fig. 6A,B). At higher core protein concentrations (100 ng core /mg mitochondrial protein), oxygen consumption from both complex I and complex II substrates was inhibited (Fig. 6B).

We further measured the effect of exogenous core protein on the mitochondrial glutathione pool (Fig. 7). In control mitochondria, 95% of the total glutathione pool was in the reduced form. Further reduction with glutathione reductase/NADH did not significantly increase GSH content. Incubation with 80 ng core protein/mg mitochondrial protein did not change the total glutathione content. However it produced a significant oxidation of the glutathione pool as evidenced by a decrease in measured GSH which could be restored by subsequent enzymatic reduction. A smaller degree of glutathione oxidation was observed at 10 ng core/mg protein and complete oxidation was produced by incubation with 0.2 mM tBOOH (Fig. 7).

ROS production in mitochondria. The effect of HCV proteins on mitochondrial ROS production was assessed using the ROS-sensitive fluorescent probe, DCFDA. Figure 8A shows that ROS production during state 4 respiration was increased in transgenic mitochondria in the presence of complex I but not complex II substrates. ROS production was completely blocked by the complex I inhibitor, rotenone (Fig 8B). In the presence of the complex II substrate, succinate, total O_2 consumption was greater (Fig. 5 B vs. D) but ROS production was less and was not increased in transgenic mitochondria. These data indicate that complex I is the primary source of the increased ROS production in HCV transgenic

mitochondria. The uncoupler FCCP reduced ROS under all conditions as previously reported (39;40), however the transgenic mitochondria retained increased complex I ROS production (Fig. 8C). Incubation of mitochondria for 5 min with recombinant core protein prior to addition of FCCP had similar effects on ROS production as did the HCV transgene expression (Fig. 8D). At a concentration of 10 ng/mg protein, core specifically increased complex I ROS production.

HCV core protein localization within mitochondria. To further determine if direct core protein interactions are a possible mechanism for in vivo effects, we next examined the nature of the interaction of core with mitochondria. Mitochondria isolated from HCV transgenic mice had two forms of core protein associated, 23 KD and 21 KD, possibly reflecting the nascent (1-191) and C-terminal truncated processed form (13;41;42). Figure 9A demonstrates that digestion of transgenic mitochondria with 50 µg/ml proteinase K removed the outer membrane protein, Tom20, without digesting the intermembrane space protein, cytochrome c, the inner membrane protein (core2 subunit of complex III), or the matrix protein, mtHSP70. After disruption of mitochondrial membranes with Triton-X-100, all proteins were digested by the protease. Incubation of control mitochondria with exogenous core protein also resulted in protease labile mitochondrial association (Fig. 9B). Therefore, under these conditions core protein behaved as an outer membrane associated protein.

Effects of core on mitochondrial Ca²⁺ uptake. One possible mechanism for how interaction of core protein with the outer membrane might affect mitochondrial redox state would be if it altered mitochondrial Ca²⁺ uptake (43). To examine this possibility we used the fluorescent Ca²⁺ indicator, Rhod-2, to determine the effect of core protein on intramitochondrial Ca²⁺. Fig. 10A demonstrates that core protein had no effect on mitochondrial Ca²⁺ content as long as mitochondria were incubated in standard isolation buffer without added Ca²⁺. Upon addition of Ca²⁺ to the mitochondria, however, core significantly increased total intramitochondrial Ca²⁺ content. As expected, Ca²⁺ uptake in this system was

completely prevented by dissipation of the mitochondrial $\Delta\Psi$ with FCCP.

We further measured the effect of core on Ca²⁺-induced ROS production in isolated mitochondria. Ca²⁺ addition to isolated mitochondria greatly increased ROS production and this effect was further increased by core protein. The Ca²⁺ chelator, BAPTA-AM, largely prevented the Ca²⁺ induced increase in ROS production and diminished the effect of core protein (Fig. 10B).

DISCUSSION

Chronic HCV infection is associated with excess oxidative stress within the liver and several different experimental models of HCV protein expression reproduce this finding. HCV transgenic mice have increased hepatic lipid peroxidation (9;11), and HCV core protein expression in hepatoma cells results in an increase in mitochondrial reactive oxygen species (ROS) production (9;10). However, the details of how the core protein alters mitochondrial function are unknown.

In order to specifically determine the nature of the mitochondrial abnormalities, we developed a new line of HCV transgenic mice. These mice express the viral proteins core, E1, E2, and p7 on a C57BL/6 background and offered us the ability to study mitochondrial function in hepatocytes from native liver. Although these mice lack viral replication, they have the advantage of expressing viral proteins in liver at levels that are characteristic of the disease in humans. The expression of core protein (0.59 pg/µg) is well within the range of 0-2 pg/µg determined in clinical specimens with the identical ELISA kit and standards (J. Liang, personal communication). Liver histology was normal making these mice similar to patients infected with the 1b genotype in that the presence of viral proteins alone does not result in massive steatosis (44;45).

Our results show that HCV protein expression caused an oxidative mitochondrial phenotype characterized by oxidation of both the mitochondrial glutathione and pyridine nucleotide pools (Fig. 3). Liver mitochondria from transgenic mice displayed a specific defect in complex I mediated electron transport and an increase in

ROS production. There were no abnormalities in complex II/III mediated oxidation of succinate. Protease digestion studies showed that core protein was directly associated with the mitochondrial outer membrane. When incubated with normal mitochondria in vitro, core protein also caused increased Ca^{2+} entry, increased ROS production, glutathione oxidation, and reduced complex I activity. It is thus likely that core protein, like multiple other viral proteins (46), has important mitochondrial effects.

Mitochondrial ROS generation can occur at either complex I or complex III (47-49) and our data demonstrate that complex I, and not complex III is the source of the core-induced ROS. Direct ROS production during state 4 respiration was increased in transgenic mitochondria only in the presence of complex I substrates, it was inhibited by the complex I inhibitor rotenone, and it was associated with decreased net electron transport activity of complex I. While complex III mediated ROS production from glutamate/malate would also be inhibited by rotenone, core-protein induced ROS production was not detected when succinate was used as substrate and thus did not originate from complex III.

Complex I ROS production has been reported to be either inhibited (50-52) or sometimes increased by rotenone (48;53;54). The variability of the effect of rotenone on complex I associated ROS production could reflect the multiple possible sites within complex I at which superoxide can be formed or could reflect different ROS detoxification at these sites (55). We readily detected an increase in succinate induced ROS after adding antimycin A (data not shown) suggesting that had the ROS source primarily been generated from complex III it would have been observed. The data thus support complex I as the site of ROS generation.

Accumulating evidence now clearly demonstrates the association of core protein with mitochondria. Core protein is located in mitochondria in transgenic liver, in several different cell lines expressing this protein, and in hepatoma cells bearing genomic-length replicons of HCV (9;18;19). Two recent studies have shown that mitochondrial core protein is specifically associated with the outer membrane (18;19) and our studies reported here confirm that this is the case in transgenic mouse liver as well. Importantly,

Schwer et al (18) demonstrated that core protein associates with the mitochondria associated membrane (MAM) fraction, a point of close contact between ER and mitochondrion. The finding that incubation of control mitochondria with recombinant core protein at concentrations similar to those associated with mitochondria in vivo, oxidized the mitochondrial glutathione pool and reproduced specific complex I changes argues that it is this direct interaction that produces the effects.

Separate events may be responsible for initiating and sustaining the core-induced increase in mitochondrial ROS production. The present study shows that core protein increases Ca^{2+} entry into isolated mitochondria with a subsequent increase in ROS (Fig. 10). This suggests that an increase in mitochondrial Ca^{2+} might be the initiating event. Increased mitochondrial Ca^{2+} has been previously observed to increase ROS production, possibly by stimulating electron flow in the respiratory chain, or altering structure of electron transport complexes (see (43;56) for reviews). At the present time the mechanism of how core increases mitochondrial Ca^{2+} uptake is not known, but direct effects on mitochondrial Ca^{2+} transporters or effects on ER-mitochondrial interactions are both possible.

Subsequent to the Ca^{2+} -induced increase in ROS production, complex I may play a role in sustaining and amplifying increased mitochondrial ROS production. Complex I is the site most sensitive to oxidative damage of the electron transport carriers and inhibition of complex I occurs during the early stages of mitochondrial damage (57). Specific inhibition of complex I has previously been reported to be a consequence of oxidation of the mitochondrial glutathione pool and consequent glutathionylation of complex I subunits (58). Murphy and colleagues have observed that glutathionylation decreased complex I activity and increased complex I ROS production. Glutathionylation was reversed by treatment with DTT, but there was no recovery of complex I activity. These findings are strikingly similar to the effects of HCV core protein on liver mitochondria. It is thus possible that core-induced GSH oxidation and consequent complex I glutathionylation can sustain and amplify the oxidized mitochondrial environment.

Net oxidation of the glutathione pool could result either from increased GSH oxidation or decreased GSSG reduction. While we did not observe any effect on glutathione reductase activity or total nicotinamide nucleotide content, the increase in GSSG was associated with a decrease in NADPH. Since the mitochondrial NADPH pool is maintained primarily by the activity of several systems such as nicotinamide nucleotide transhydrogenase, isocitrate dehydrogenase, and malate dehydrogenase (59), it is possible that changes in the activities of these enzymes, in addition to Ca²⁺ induced ROS production, could contribute to net oxidation of the glutathione pool.

It has previously been shown that several HCV proteins, including core protein (60), can contribute to a state of ER stress (61) and this may secondarily alter mitochondrial function via

changes in Ca²⁺ homeostasis (62). While we cannot rule out a contribution of ER stress in the transgenic mouse liver, the experiments with in vitro addition of core protein indicate that direct mitochondrial effects are present as well.

In conclusion, our study shows that HCV core protein localizes to mitochondria, associates with the mitochondrial outer membrane, increases mitochondrial Ca²⁺ uptake, and causes oxidation of the glutathione pool. This change in mitochondrial redox state inhibits complex I activity, further increases ROS production and can create positive feedback loop (58). Mitochondrial GSH depletion or oxidation is associated with enhanced liver injury in alcoholic, toxic, and inflammatory liver diseases (63), and thus could have significant effects on response to inflammation and the development of progressive liver disease in chronic Hepatitis C.

ACKNOWLEDGEMENTS

We thank Dr. S. Watowich for providing core protein, S. Lemon, J. Liang and Y. Li for helpful discussions and I. Boldogh and T. Qian for critical advice.

Supported by grant AA12863 from the National Institute on Alcohol Abuse and Alcoholism.

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FIGURE LEGENDS

Figure 1. Characteristics of SL-139 HCV Transgenic Mice (A) Schematic representation of transgene vector. (B) Northern blot analysis revealing the presence of two specific transcripts of 3.4 and 4.2 kb. Marker bands are shown for molecular weight comparison.

Figure 2. HCV core protein expression in transgenic mouse liver and mitochondria. Immunoblot demonstration of HCV core protein expression in liver mitochondria .CON, control mice; TgM, transgenic transgenic mice; L,liver lystate; M, mitochondrial lystate; (A) Each lane was loaded 100 µg or either total lysate or mitochondrial protein. Two forms of core protein were detected in transgenic but not control samples. HCV core protein in mitochondria was approximately 4 fold more abundant in mitochondria than in liver lysate. (B) Purified HCV core protein (1-179) was added to control

mitochondrial lysate (10 ng or 5 ng/mg mitochondrial protein) and compared to two separate mitochondrial lysates.

Figure 3. Glutathione content in HCV transgenic mouse liver. Glutathione content was measured in freshly isolated whole liver homogenate (**A,B**) or mitochondrial fractions (**C,D**) as described in Methods. Total glutathione (GSH + GSSG) was expressed as GSH equivalents (**A,C**). Reduced glutathione (GSH only) is shown in panels **B,D**. Values in transgenic liver samples were compared to those in corresponding control liver samples (n=3-6, **P<0.01). Baseline values for total glutathione in control samples were 36.6 ± 1.9 nmole/mg protein for whole liver, and 8.2 ± 1.2 nmole/mg protein for mitochondria.

Figure 4. Measurement of oxygen consumption. P:O ratio (**A**) and maximum oxygen consumption after treatment with 5 μ M FCCP (**B**) were measured after incubation of control or transgenic mitochondria with 5 mM glutamate and 5 mM malate (complex I substrates). P:O ratio (**C**) and maximum oxygen consumption (**D**) were similarly measured with 5 mM succinate (complex II substrate). Where indicated, mitochondria were preincubated for 5 min with 100 μ M tBOOH at 25°C (n=8 without tBOOH, n=5 with tBOOH, *P<0.05, **P<0.01).

Figure 5. Mitochondrial complex I and complex III activity. (**A**) Complex I activity (NADH-decylubiquinone reductase) was measured in submitochondrial fractions prepared from control (N=11) or transgenic (N=7) mouse liver before (DTT-) or after incubation with 100 μ M dithiothreitol (DTT+) (n=4). (**B**) Complex III activity (ubiquinol cytochrome c reductase, n=6). ** p<0.001, * p<0.05

Figure 6. Effects of HCV core protein on respiration in control mitochondria. The effect of recombinant HCV core protein on (**A**) P:O ratio and (**B**) maximum O₂ consumption was determined after incubation of control mitochondria (1 mg) with different quantities of HCV core protein at 25°C (n=3 mitochondrial preparations for each condition) *P<0.05, **P<0.01), Glu/Mal, glutamate and malate; Suc, succinate.

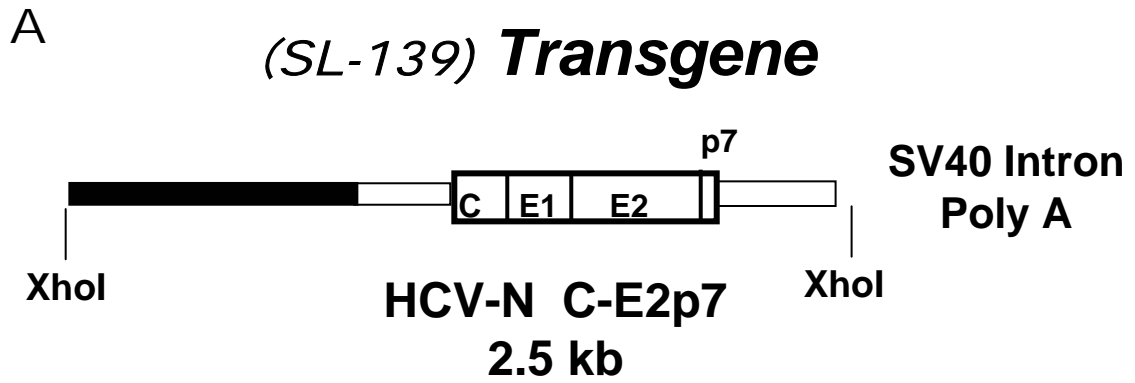
Figure 7. Effect of in vitro core protein incubation on mitochondrial glutathione. Freshly isolated mitochondria from control livers were incubated in PBS for 5 min at 25°C with no addition (control), 10 ng/mg protein HCV core protein (10 core), 80 ng/mg protein HCV core protein (80 core), or 0.2 mM tBOOH (tBOOH). Total glutathione was measured directly by the DTNB recycling assay as described (black bars). Reduced glutathione samples were measured either before (striped bars) or after (grey bars) reduction of the glutathione pool with glutathione reductase and NADPH. *P<0.05.

Figure 8. ROS production in isolated mitochondria. (**A**) ROS production was measured by the change in DCF fluorescence under conditions of state 4 respiration. (**B**) Effect of rotenone (1 μ M) on ROS production. (**C**). Effect of uncoupler (5 μ M FCCP) on mitochondrial ROS production. (**D**) Purified core protein (10 ng/mg protein) was incubated with control mitochondria under the same conditions as in Figure 7C (n=4 individual experiments. *P<0.05, **P<0.01), G/M, glutamate and malate; S, succinate; (-), no substrate.

Figure 9. Localization of HCV core protein in mitochondria. (**A**) Mitochondria were isolated from SL-139 transgenic mouse liver and treated with 50 μ g/ml proteinase K in the presence or absence of Triton X-100 and immunoblotted for core protein and specific marker proteins (Tom20, outer membrane; cytochrome c, intermembrane space; complex III core II, inner membrane; mtHSP70, matrix). (**B**) Control mitochondria were incubated with either 10 or 100 ng/mg protein HCV core protein for 5 min at 25° and then treated with proteinase K with or without Triton X-100 as described.

Figure 10. Effect of core protein on Ca²⁺ uptake and ROS production in isolated mitochondria. (A) Ca²⁺ uptake was measured by Rhod2 fluorescence before or after incubation with 125 μM Ca or 125 μM Ca with 5 μM FCCP. Where indicated mitochondria were first treated with core protein (10 ng/mg protein). (B) ROS production was measured by the change in DCF fluorescence as in Fig. 8. Data was normalized to the baseline signal for control mitochondria. Each measurement was repeated in 6 separate wells for each of 3 independent mitochondrial preparations from separate mice. *P<0.05, **P<0.01).

Figure 1



B

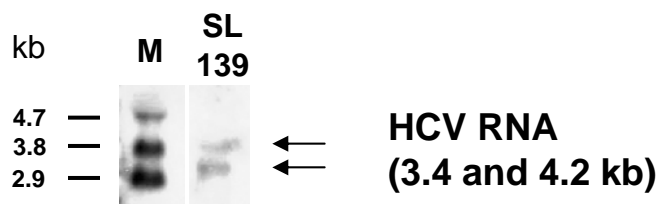


Figure 2

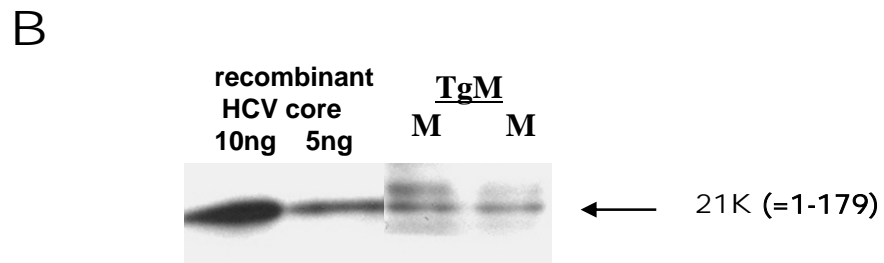
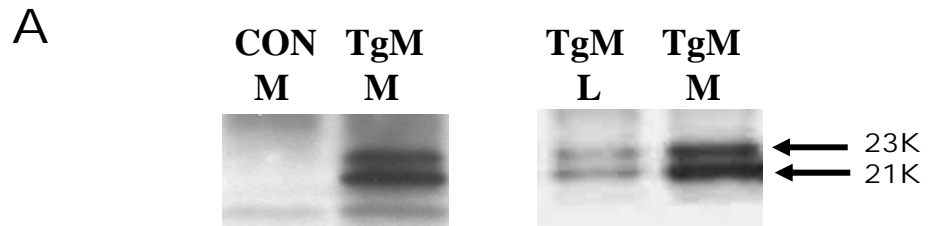
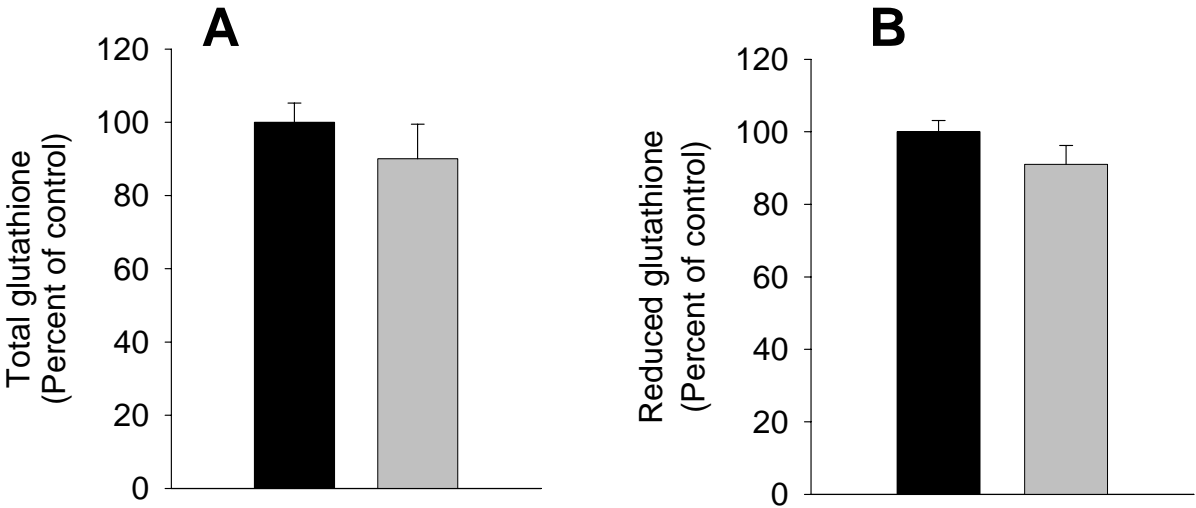


Figure 3

Control
Transgenic

Whole liver



Mitochondria

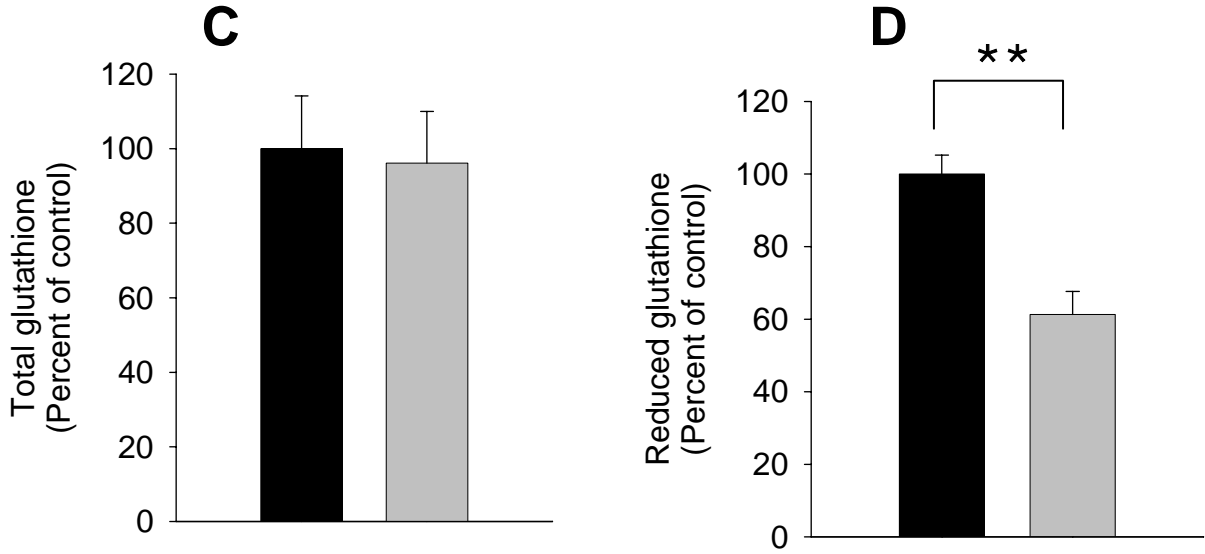
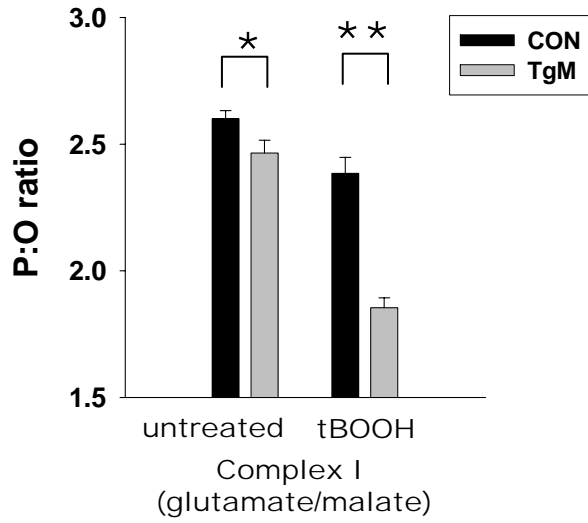
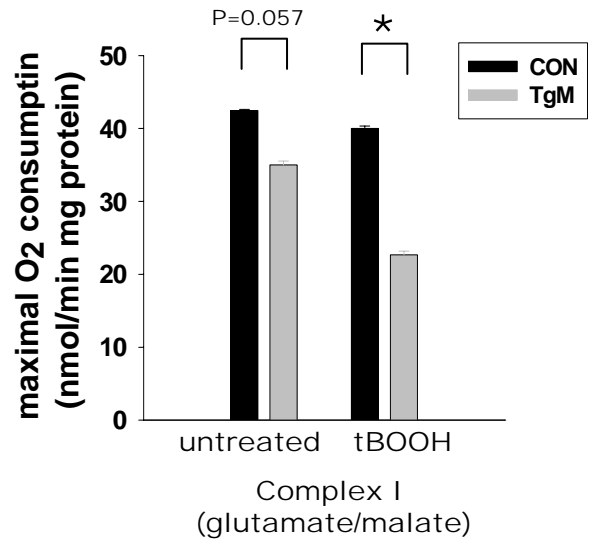


Figure 4

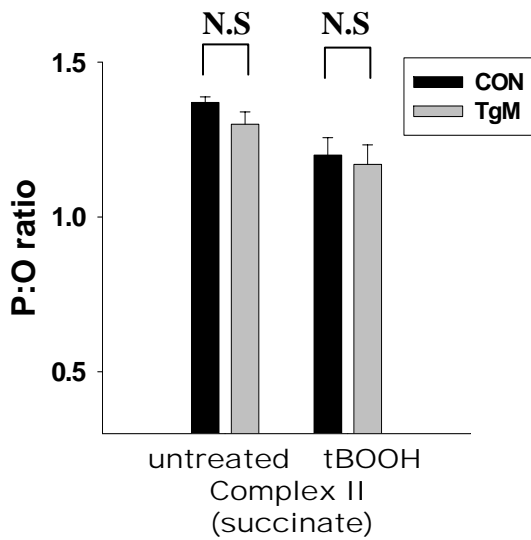
A



B



C



D

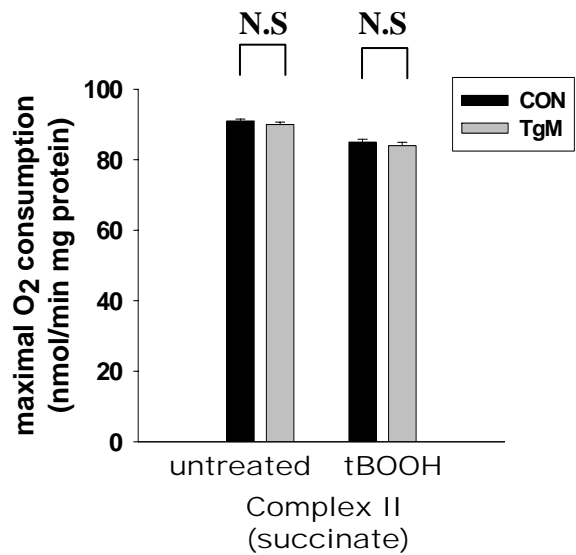


Figure 5

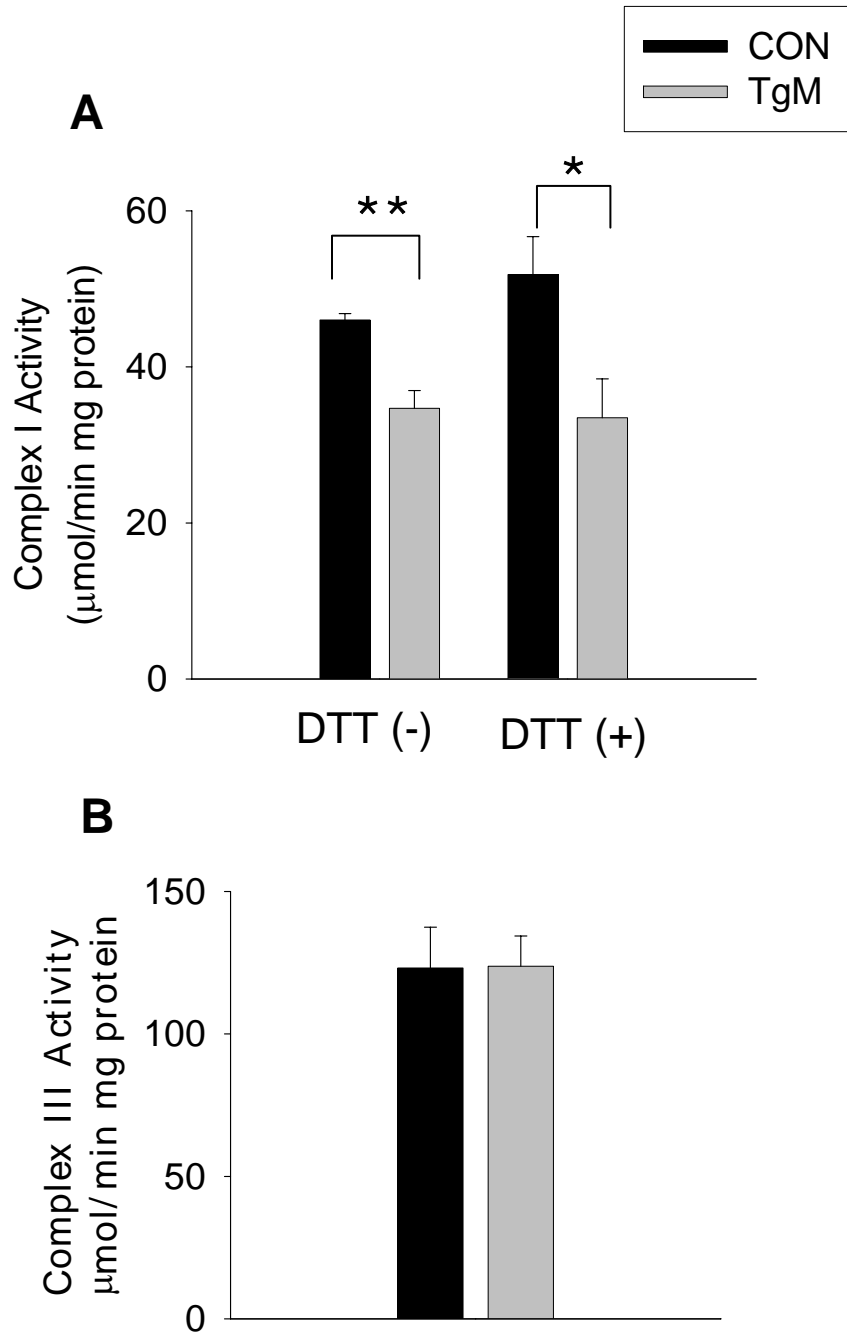


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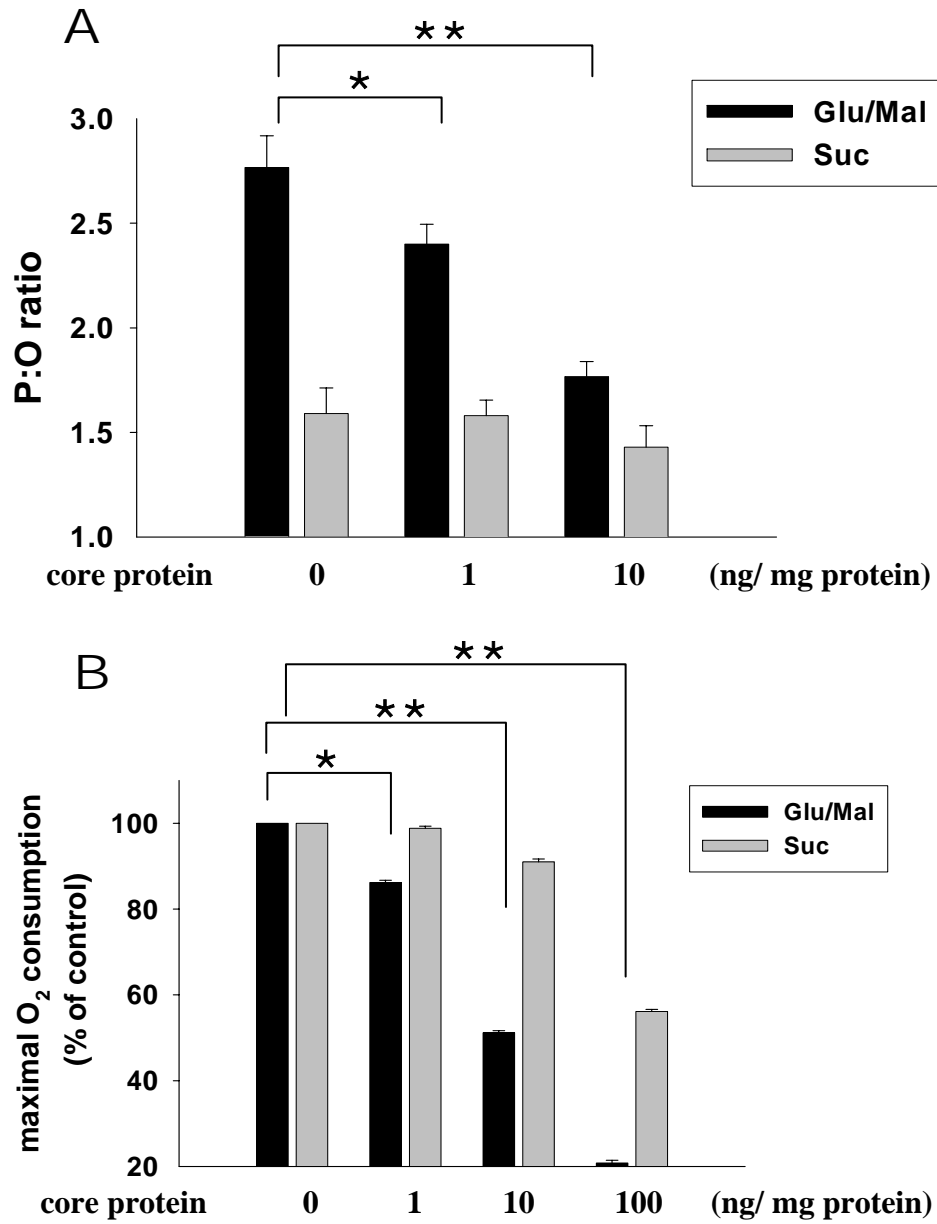


Figure 7

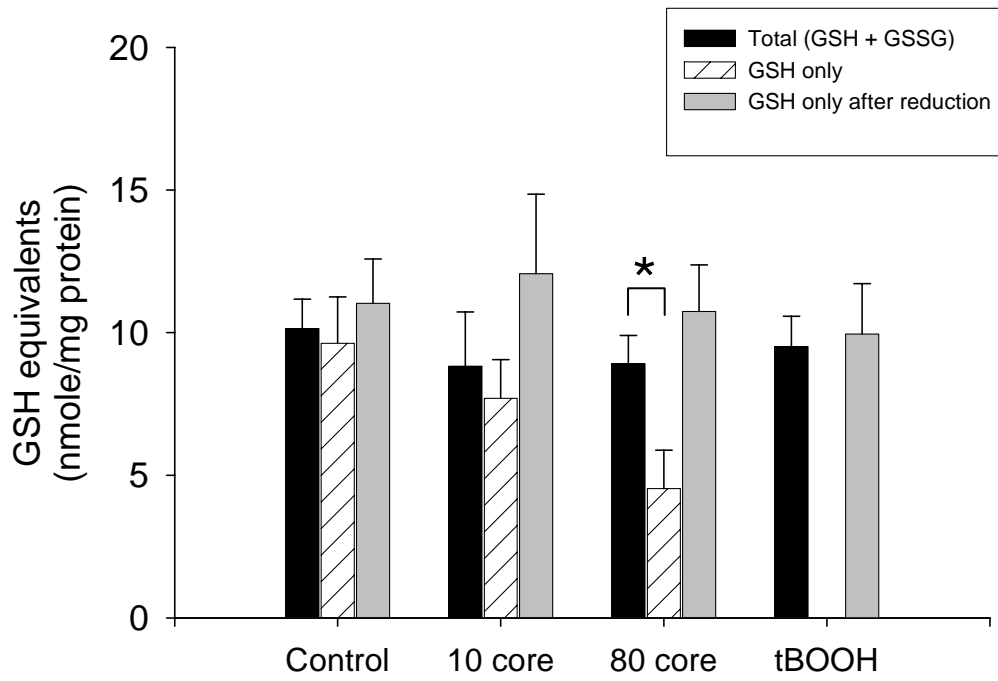


Figure 8

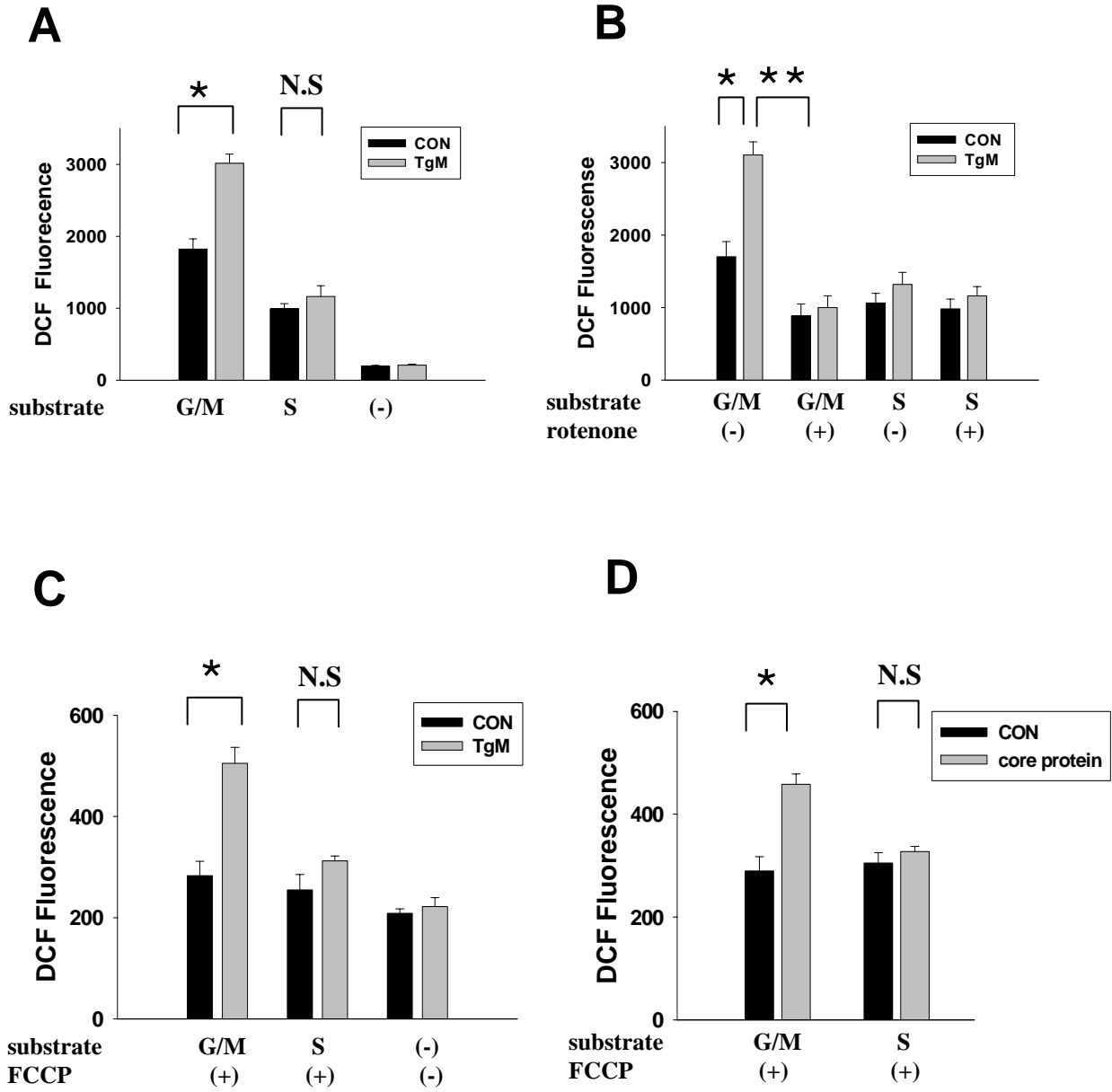
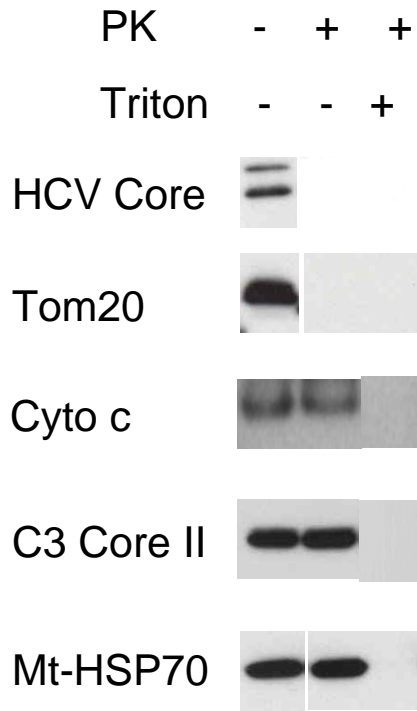


Figure 9

A



B

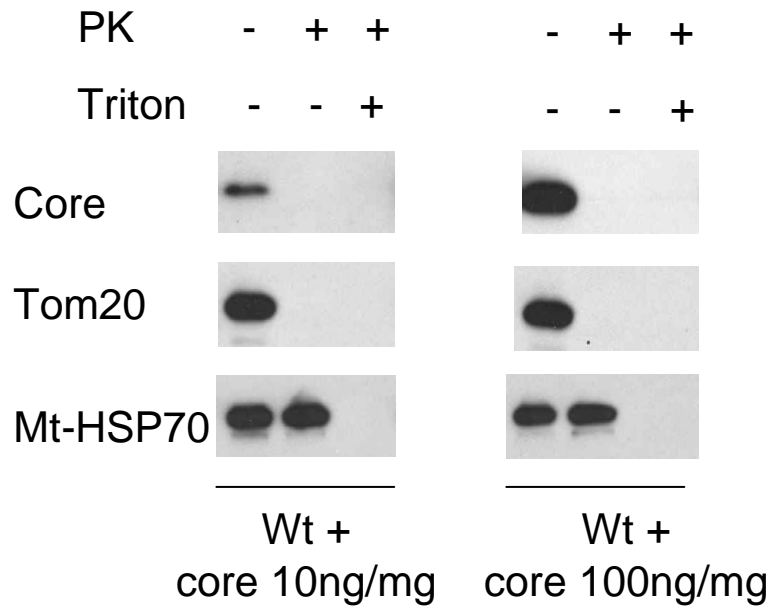
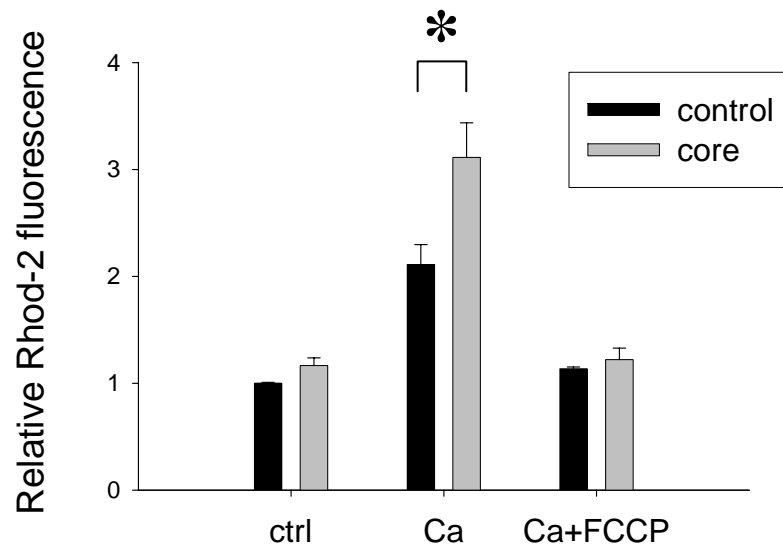


Figure 10

A



B

