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Nox1 and Nox4 enzymes are persistently elevated in human hepatocytes producing infectious hepatitis C virus

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Quantitative and Systems Biology

by

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The Thesis of Nabora Soledad Reyes de Mochel is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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Dedication

This thesis is dedicated to my mother, Porfiria Virrey. In honor of the struggles she overcame while raising my sisters and I, in this foreign country. All the hard work I put into this thesis is a symbol of my upbringing. My mother encouraged me to strive for the impossible and never lose sight of what I desire. Gracias madre querida, te amo.

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List of Abbreviations

Hepatitis C virus (HCV), reactive oxygen species (ROS), reactive nitrogen species (RNS), superoxide dismutase (SOD), glutathione (GSH), oxidized glutathione (GSSG), diethyldithiocarbamate (DDC), H_2-dichlorofluorescein diacetate (DCF-DA), nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H oxidase or NOX), (DPI), Chronic granulomatous disorder (CGD), internal ribosomal entry site (IRES), open reading frame (ORF), transforming growth factor-beta (TGF-β), Toll-like receptor 4 (TLR4), nuclear factor kappa B (NFκB), hepatocellular carcinoma (HCC), nitric oxide synthase (iNOS), alanine aminotransferease (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), enzyme-linked immunosorbent assay (ELISA), superoxide ($O_2^{\bullet -}$), hydroperoxyl ($HO_2^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl, $RO^{\bullet}$, hydroxyl radical ($^{\bullet}OH$), carbonate radical $(CO_3^{\bullet})^-$, carbon dioxide radical ($CO_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), ozone (O_3), electron transferring flavoprotein (ETF), glyceraldehyde-3-phosphate dehydrogenease (GAPDH), human hepatoma (huh7) cells, nitric oxide (NO), diphenyliodonium (DPI), Dulbecco's phosphate buffered saline (DPBS), horseradish peroxidase (HRP), bicinchoninic acid (BCA), optical density (OD), human umbilical vein endothelial cells (HUEVCs), thioredoxin (Trx), glutaredoxin (Grx)

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ABTRACT OF THE THESIS

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Abstract

Hepatitis C virus (HCV) is an etiologic agent of severe liver diseases in humans. HCV infection has been associated with severe alterations of the host redox status, and oxidative stress has been identified as a key mechanism of HCV-induced pathogenesis. Nevertheless, the source of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) during HCV infection is still incompletely characterized. Recently, hepatocytes have been found to express Nox family enzymes, but it is unknown if these proteins participate in HCV-induced oxidative stress. We found that Nox4 and Nox1 mRNA's were persistently elevated in Huh7 cells that produced infectious HCV particles, compared to mock-transfected controls. Duox2 mRNA was only transiently elevated with HCV. The same Nox/Duox mRNAs were also increased in telomerase-reconstituted primary human hepatocytes transfected with HCV RNA. In contrast, subgenomic HCV RNA did not increase Nox/Duox mRNA's, suggesting that the structural genes or the production of infectious virions is necessary. In addition, Nox4 and Nox1 protein contents were elevated in HCV RNA-transfected and -infected cells as well as human liver. HCV increased the oxidation of H_2-dihydrodichlorofluorescein diacetate generated from incubation of cells with (DCF-DA) that was sensitive to diphenyl iodonium, an inhibitor of flavoenzymes including Nox enzymes. Also, the levels of H_2O_2 and 2-OH-E+ were elevated in HCV-JFH1 RNA transfected hepatocytes, and siRNAs to Nox enzymes decreased H_2O_2 level. Finally, confocal laser scanning microscopy and subcellular fractionation studies showed that Nox4 was prominent in the nuclear compartment of these cells. Therefore, hepatocyte Nox proteins may act as an endogenous source of oxygen- centered reactive species during chronic HCV infection and are likely to contribute to the pathogenesis.

Chapter 1: Introduction

Hepatitis C virus

Hepatitis C virus (HCV) is a *hepacivirus* belonging to the *Flaviviridae* family. Although it shares some similarities with yellow fever virus, and other members of the RNA genome family, HCV is classified with its own genus. Unlike DNA viruses, HCV undergoes significantly higher mutation rates that occur predominantly in the viral envelope protein region (E2). This hinders the development of effective prophylactic vaccines and antiviral therapy. HCV was discovered in 1989 as the etiologic agent of non-A/non-B hepatitis. The majority of the infected individuals acquired the virus from blood transfusions (prior to 1990 when screening of blood before transfusions was not mandated) and contaminated blood products (49). Today, HCV is transmitted by two major routes: 1) needlestick injuries in drug use and in health care settings and 2) infant exposure to an HCV infected mother (8). HCV-induced liver damage is commonly diagnosed by the detection of high levels of alanine aminotransferease (ALT), aspartate aminotransferase (AST), alkaline phosphatase, lactate dehydrogenase (LDH), and bilirubin in the blood (40).

The viral genome is an RNA strand of positive polarity with an internal ribosomal entry site (IRES) at the 5' end and an untranslated region (UTR) of 300bp at the 3' end. The open reading frame (ORF) is 9,000 base pairs (bp) and encodes for a polyprotein which is cleaved to generate individual viral proteins (47). Translation begins with the structural proteins, Core, E1, E2, p7, and non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Serine-, thiol- and zinc- metalloproteinases produce essential proteins for viral replication and assembly (31, 47). Core, E1, and E2 participate in viral

genome packaging and egress which occurs via host-secretory pathway. In addition, translational frameshift occurs within the core-coding translational region which gives rise to F/ARF protein, the function of which is still unknown. HCV p7 protein is thought to possibly serve as virion-particle chaperone or an ion channel, whereas, NS3 is a serine proteinase that cleaves the HCV polyprotein (31). NS5A has been implicated to decrease the IFN-α anti-viral activity and to induce transforming growth factor-beta (TGF-β) by activating Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NFκB) (43). NS5B, is the RNA-dependent RNA polymerase; that assembles with the rest of the NS proteins to form the replication complex. The replication complex synthesizes the (-) strand from a (+) strand. The process begins with translation of key viral catalysts and regulatory proteins that effectively converts the human hepatocyte into a virion-producing machine. HCV has been known to primarily infect human and chimpanzee hepatocyes. Scientists have also produced immunocompromised mice that have human hepatocytes and can sustain HCV (22). It has been proposed that viral entry occurs by CD81 or LDL receptor-mediated endocytosis. E2 has been implicated in binding to the tetraspanin molecule CD81 found in hepatocytes and B lymphocytes (47).

HCV and oxidative stress

Worldwide, about 170 million people are estimated to have been infected with HCV, with four million of them residing in the United States. Upon viral infection, about 15% of the infected individuals can clear the infection. The remaining 85% of infected population will develop chronic infection that can result in liver cirrhosis, steatosis, hepatocellular carcinoma (HCC) and eventually liver failure. Despite efforts, the host

and viral mechanism that lead to hepatic pathogenesis are yet to be completely elucidated.

However, during HCV infection, there is evidence of increased inflammation with the recruitment of neutrophils (20). Long term hepatic inflammation eventually damages the liver while elevating hepatic enzyme levels in the blood. In addition, an increase of reactive oxygen/nitrogen species (ROS/RNS) has been observed along with a decrease in antioxidants, and oxidative/nitrosative stress has been identified to be a potential key player in HCV induced hepatopathogenesis (41). Reports of the induction of nitric oxide synthase (iNOS) genes indicate that nitric oxide ($NO^{\cdot}$) synthesis is also increased during HCV infection (13,15,33). $NO^{\cdot}$ reacts with ROS, specifically with superoxide anion to create peroxynitite. Interestingly, HCV infected patients also showed a decrease in GSH levels and an increase in glutathione disulfide (GSSG); thus, indicating that there is a sufficient amount of GSH being oxidized in patients with HCV infections (41).

DNA oxidation is another marker of oxidative tissue damage that has been frequently detected in hepatitis C patients. Guanine often serves as the site of base mutations (21). The oxidation of deoxyguanosine into 8-hydroxydeoxyguanosine has been detected in urine, plasma serum, and tissue from patients infected with HCV (3,12, 45), thus suggesting an increased generation of ROS/RNS during HCV infection. DNA oxidation can be detected using an *in vitro* assay such as an enzyme-linked immunosorbent assay (ELISA); which utilizes antibodies with specific affinity for 8-hydroxydeoxyguanine.

ROS/RNS

ROS is the term used to classify a group of oxygen-derived free radicals and reactive non-radicals in high oxidation states. Superoxide ($O_2^{\bullet -}$) is composed of two oxygen molecules with an unpaired electron; which makes the molecule susceptible to both reductive and oxidative reactions. The production of other free radicals begins with generation of superoxide, and these are hydroperoxyl ($HO_2^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl, $RO^{\bullet}$, hydroxyl radical ($^{\bullet}OH$), as well as carbonate radical $(CO_3^{\bullet})^-$, and carbon dioxide radical ($CO_2^{\bullet -}$). Other non-radical reactive oxygen species include hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and ozone (O_3) (16). All oxygen-centered reactive species are referred to as ROS; however not all ROS are free radicals. These radicals react with other molecules at high rates. Superoxide ($O_2^{\bullet -}$) is eliminated at the diffusion-limited rate of 10^9 $M^{-1}sec^{-1}$ and hydroxyl radical has a half life of 10^{-9} sec and it reacts with any molecule within a 30 Å radius (4). The presence of ferrous iron (Fe^{2+} or iron II) and H_2O_2 creates the possibility of Fenton chemistry to occur yielding ferric ion (Fe^{3+} or iron III,) hydroxyl ion and a hydroxyl radical. Ferric ion may also be reduced by hydrogen peroxide to form Fe^{2+}, $HO_2^{\bullet}$ and H^+ (16). The onset of free radical formation triggers the production of other radicals, creating an environment favoring oxidation/reduction reactions.

Antioxidants

Eukaryotes adapted to an introduction of 21% oxygen in the atmosphere by evolving antioxidant defenses that allowed them to quickly remove superoxide and hydrogen peroxide and thus create a more stable microenvironment. Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\bullet -}$. In humans there are three isoforms

of SOD: SOD1 found in cytosol, SOD2 (52) in mitochondria, and SOD3 found extracellularly (53). Another important antioxidant that defends the cellular microenvironment from free radicals is glutathione (GSH). Glutathione is a tri-peptide that is predominantly found in its reduced form within healthy cells. GSH peroxidases use GSH to remove H_2O_2 and during this process GSH is oxidized to form the disulfide-bridged dimer, GSSG. GSSG is reduced back by glutathione reductase. Increased levels of GSSG indicate that there is a substantial increase in the turnover of GSH into GSSG that exceeds the rate of reduction of GSSG back to GSH. The reduction of GSSG back to GSH allows for conversion of harmful radicals to occur by facilitating the reduction of other proteins. Synthesis of glutathione in cells also increases during oxidative stress as an adaptive response. In addition, thioredoxin (Trx) and peroxiredoxin are also part of important antioxidant systems that maintain the redox homeostasis (36). Thioredoxin and glutaredoxin (Grx) get reduced using NADPH and are located in the cytoplasm of all living cells (18). Finally, catalase and bilirubin also contribute to the overall cellular microenvironment's redox state and function to eliminate ROS (16).

Sources of ROS/RNS during HCV infection

Radicals are generated when a covalent bond is homolytically cleaved in a closed-shell molecule or when a closed-shell molecule loses or gains one electron. There are many ROS producers in cellular systems (16). Xanthine oxidase generates ROS and plays an important role in uric acid metabolism. This enzyme is found in the liver of healthy individuals and detectable in the blood of those who have liver damage.

Free radical formation can also occur at complexes I and III, and coQ pool of the mitochondria (16).

Currently in the HCV field, the general consensus is that HCV core protein causes mitochondrial membrane depolarization and ROS production at complex I (23). Several groups have also demonstrated the increase of inducible iNOS by HCV, which was then suggested to increase DNA damage by $NO^{\cdot}$ reacting with mitochondrial ROS (13,14). The ROS produced in the mitochondria are likely to increase oxidative stress during HCV infections; however it does not adequately explain how genomic DNA oxidation occurs in a nitric oxide-dependent manner (33). Peroxynitrite, for example, is highly reactive and will tend to react with whatever molecule is close to its site of generation; therefore, for peroxynitrite to damage the DNA directly, it will need to be generated immediately adjacent to the DNA. In addition, the presence of antioxidant enzymes that rapidly remove superoxide in and out of the mitochondria makes it highly improbable for superoxide that is generated in mitochondria to diffuse to and through nuclear envelopes to react with nitric oxide to oxidize the genomic DNA in a nitric oxide-dependent manner. This leads to the question of how DNA is getting damage by ROS/NOS if the source of superoxide is located in the cytoplasm. Therefore, the location of the proposed ROS source needs to be further investigated. Given the high reactivity of ROS, the source of these highly reactive species responsible for the oxidation of the host nuclear DNA is most likely located close to cell nucleus.

NADPH oxidase

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or Nox enzymes were known as cyanide-insensitive pyridine nucleotide-linked oxidases. These are enzymes whose sole purpose is to catalyze the production of $O_2^{\bullet-}$ and H_2O_2, are found in different cell systems that include vascular, renal, pancreatic, and immune cells (neutrophils, eosinophils, macrophages, and lymphocytes) (1,17, 24, 25,27, 42,44). One of these enzymes was primarily identified in patients who suffered from chronic granulomatous disorder (CGD). These patients suffered from not being able to clear simple bacterial and fungal infectious due to a defect in the phagosomes of polymorphonuclear cells (PMN) (17). The neutrophils were able to engulf foreign pathogens; however the pathogens were not cleared or dissolved creating a ball of immune cells known as granulomas (20). Now, it's understood that phagosomes formed after engulfing of foreign pathogens were unable to utilize Nox2 to generate superoxide to attack and damage the pathogen cell wall barrier (17). In contrast to the previously accepted theory that NOX proteins utilized NADH instead of NADPH for the electron transfer, the findings of David C. Hohn and Robert L Lehrer demonstrated that NADPH was employed by the multi-complex oxidase (17). The prototype NADPH oxidase mentioned above is what is now referred to as Nox2.

In 1999, MOX1 (Nox1) was identified and partially characterized. Shortly following this discovery, Nox3, Nox4, and Nox5 were identified (26). Today there are seven known isoforms of the NOX proteins: Nox 1 – 5, which transfer one electron, and Doux1 - 2 which are involved in two-electron transfer reactions (9). The prototype Nox2 is composed of six subunits that bind together activating the oxidase. The flavochrome b_{558} complex is membrane bound and consists of two proteins, $gp91^{phox}$ and $p22^{phox}$. The β-subunit ($gp91^{phox}$) shares more homology with the other NOXes because it is the most

conserved subunit. The heme NAD(P)H binding sites are located within the gp91. The $p22^{phox}$ has a carboxylic tail in the cytosol; which has been implicated to bind the $p47^{phox}$ subunit. In the cytosol, are the three proteins that make up the cyclic oxidase subunit, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ (42,48). The subunit, $p47^{phox}$, is believed to be the chaperone protein that guides the cytosolic assembled complex to the membrane. There are four crucial phosphorylation sites on the $p47^{phox}$, S303, S304, S358,and S370. When an alanine is inserted at S358 and S370, the oxidase fails to be activated (1). The putative function of $p40^{phox}$ remains controversial. Some believe that it helps keep the subunit in the inactive form, and others believe it participates in the activation of the oxidase. Although the function of the $p67^{phox}$ has not been clearly identified, it has been suggested to indirectly participate in the electron transfer (1). The interaction between $p67^{phox}$ and activated Rac dimer is believed to be crucial for the activation of the $gp91^{phox}$ subunit (48). A small GTP binding protein RAC1 binds GTP to further activate NOX. Guanine nucleotide dissociation inhibitor (GDI) inhibits Rac1 (5, 48). Since the discovery of NOX proteins, these proteins have been implicated in a number of diseases including cancers (28, 29). In addition, Nox4 has been suggested to be located in the nucleus of certain cell types (24).

Summary and hypothesis

Therefore, oxidative stress has been implicated in the DNA damage and development of hepatocellular carcinoma (HCC) in patients with HCV infection (2); however the source of ROS is not clearly been identified. As previously mentioned, there is some evidence that HCV increases mitochondrial generation of ROS that contribute to the DNA damage (10). These conclusions were reached solely based on

the core and other HCV proteins-expressing systems, which may not reflect a pathogenic pattern seen during *in vivo* HCV infection. Also, as stated above, this source of ROS does not adequately explain nitric oxide-dependent DNA damage that occurs during HCV infection.

This study, therefore, sought to identify the source of ROS proximal to the host DNA. We hypothesized that HCV activates NADPH oxidase 4, which has been found in the nucleus of other cell types (24), and other NOX protein(s) in hepatocytes. The Nox-mediated generation of ROS with corporation of RNS would facilitate DNA damage in the development of hepatocellular carcinoma (HCC). To further investigate this hypothesis, my specific aims were to: 1) analyze gene expression of NAD(P)H oxidases in cellular models infected with HCV; 2) determine the cellular location of NAD(P)H oxidase; and 3) assess enzyme activity, using hepatocytes producing HCV.

Chapter 2: Experimental Procedures

HCV Cellular Models, Cell Culture

The two cellular models used to understand HCV infections in cell culture systems; were the RNA JFH1 and DNA CG1b models. The first system uses Huh7 cells to successfully withhold an HCV-JFH1 genotype 2a infection and thus the cell line is an optimal experimental model. DNA plasmid containing T7 human promoter and the JFH1 construct was used as a template for reverse transcribing JFH1 viral RNA. The primary fetal hepatocytes with reconstituted telomerase (provided by Dr. Mark Zern) were transformed with CG1b genotype 1b in a DNA plasmid containing a human promoter and ribozymes before and after the HCV genome. Upon transfection, the host's DNA dependent RNA polymerase (pol II) transcribes the plasmid yielding viral RNA. Hepatocytes were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were kept at 5% CO_2 in a VWR incubator set at 37°C.

Infected and non-HCV infected liver tissues were donated by and acquire through the National Disease Research Interchange (NDRI).

Transfection, infection, and transformation

Huh7 cells were electroporated at 260 volts, 950 uf and 4mm (Bio-Rad Gene Pulser Xcell) with 10μg of viral RNA (JFH1) and immediately cultured to stimulate cellular uptake the viral RNA. The viral RNA was introduced to cells using lipofectamine, a lipid delivery reagent (Invitrogen). The lipid droplets bind to the viral RNA and aggregate to the hepatocyte's plasma membrane. Subsequently, endocytosis occurs and the Viral RNA is translated giving rise to viral protein and thus viral replication. The

hepatocytes require approximately three hours to adhere to the bottom of the cell culture dishes. Following this process, media was exchanged to get rid of cell debris. The media was collected and tested for HCV titer. Around 10ml of the media containing substantial virion concentration was used to infect other hepatocytes by simply adding the collected media and incubating them. The hepatocytes were collected at three time periods: 48, 72 and 96 hours. For longer time-frame experiments the media was exchanged when necessary and cells were evaluated to ensure that the infection was persistent. Transfection of genotype 1b (CG1b) DNA into primary hepatocytes was performed using liposomes (lipofectamine, Invitrogen). The DNA plasmids containing ribozymes, human promoter, and HCV genotype 1b was delivered into the nucleus where the host's Pol II created the RNA transcript with a 5' end cap and a poly A tail. As previously mentioned, HCV's 5' end contains an IRES and does not need the 5' end cap. The ribozymes will then cleave the 5' end and poly A tail allowing the viral RNA to be translated as done in a natural infection. The transient transformation was followed by a more stable transfection obtained by selection. The plasmid contains an antibiotic resistant gene which selects only for those cells containing the foreign plasmid. Stable cell lines were produced by selection using 0.5% geneticin antibiotic in DMEM. Huh7 cells were transformed with plasmids containing Nox1 and Nox4 genes. This was done using liposome and plasmids that contained an antibiotic resistance gene to ensure a persistent transfection. As control cell models, hepatocytes were electroporated without any viral RNA. Control and HCV transfected cells were processed in an identical manner. Control transformations utilized an empty vector (pDCEF) that lacked the viral component but contained the antibiotic resistance gene which allowed cells to retain the plasmids.

Quantitative Real time Reserve Transcriptase Polymerase Chain Reaction

Total intracellular RNA was collected in Trizol reagent (Sigma). First, cells were rinse with 1X PBS and scraped in trizol into microcentrifuge tubes. RNA was extracted by adding $^{1}/_{10}$ v/v of100% chloroform and ½ v/v of isopropanol, centrifugation, and removal of the supernatant. The pellet was rinsed with 70% ethanol and the collected RNA was resuspended in DEPC treated water. RNA concentrations were determined using optical density (OD) values obtained with 1μl sample using the NANO drop (ND-1000 spectrophotometer). Nox1 primers read as follows: forward 5' TTAACAGCACGCTGATCCTG 3' reverse 5' CTGGAGAGAATGGAGGCAAG 3'. Nox4 primers are as follows: forward 5' CTGGAGGAGCTGGCTCGCCAACGAAG 3' and reverse 5' GTGATCATGAGGAATAGCACCACCACCATGCAG 3' (38). Analysis was conducted using Applied Biosystems 7300 real time PCR systems and accompanying reagents. Data was normalized by glyceraldehyde-3-phosphate dehydrogenease (GAPDH).

Subcellular fractions for the location of NADPH oxidases

Subcellular fractionation into nuclear and cytoplasmic fractions was performed using the NE-PER (Pierce) kit and a protocol published by *Dignam et al.* (6). In brief, 1X PBS rinsed and centrifuged cell pellet was re-suspended in 400μl of a hypotonic buffer (10mM HEPES pH 7.9, 0.5% IGE PAL, 2mM MgCl2, 10mM KCl, 0.1 MM EDTA, protease inhibitor and 1.0 mM DTT) and incubated on ice for 10 minutes. Samples were centrifuged for 6 minutes at 14,000 rpm and the cytoplasmic fractions collected. To remaining pellet, 100μl of high salt buffer (50mM HEPES pH7.9, 3mM NaCl, 50mM, KCl, 0.1 EDTA, protease inhibitor and 1.0mM DTT) was added, and the pellets were incubated on ice for 30 minutes. This process was followed by centrifugation at 12,000

rpm for 2 minutes and collection of the nuclear fraction. Fractions were analyzed by western blotting.

Western Blotting protein Analysis

Cells were collected after experimental treatments (ex: siRNA), into microcentrifuge tubes using 1X PBS and rubber cell scrapers. Cell pellets were sonicated in Laemmli buffer (1X upper tris, 208 mM SDS, 20% glycerol, 10% 2-mercaptoethanol and deionized water) three times in segments of 15 seconds using Brandson sonifier 150. Samples were kept on ice immediately after each sonication, centrifuged and transferred to new microcentrifuge tubes. When doing western blots with other buffers (other than laemmli buffer) 5-10μg of samples was used for analysis. The protein concentration was examined using bicinchoninic acid (BCA) reagent (Pierce). To samples sonicated in laemmli buffer an approximate amount of the buffer was added and data was normalized by β-actin. Samples were ran using Novax 10-20% Tricine gels 1.0mm with 10 or 15 well capacity (depending on the sample protein concentration) in 1X SDS running buffer at 120 volts for about 1 ½ hours. The gel was rinsed with transfer buffer (10.5g tris base, 33.7g glycine, and 25% methanol) and set for over night transfer into a PVDF membrane (Millipore) using 37 millivolts. Next day, membranes were rinsed with TBS (5 ml of 1M Tris, 15ml of 5M NaCl in deionized water) and blocked in 5% milk in TBS to eliminate unnecessary background. Primary antibody was done over night at 4°C and the concentration ranged from 1:200 to 1:1000 depending on the antibody stock concentration. Secondary HRP conjugate antibody and exposure was done on third day of experiment, concentrations ranged from 1:2500 to 1:5000 dilution in 5% milk in TBS-T (5ml of 1MTriz, 15ml of 5M NaCl and 0.25ml of Tween-20 in deionized water). The membranes were exposed with chemiluminescence

reagents from ECL kit (General healthcare). After applying the 1:50 dilution of solution A and B, membranes were blot using blotting paper and image were taken using Kodak Imager (2000). Antibodies for Nox1 and Nox4 were a kind gift from J. R. David Lambeth. Calnexin, *pan*-cadherin, and GAPDH antibodies were used for cytoplasmic markers. Lamin A/C, and HDAC1 were used as nuclear markers. All other antibodies were purchased from Santa Cruz biotechnology.

Immunofluorescence of human hepatocytes and liver tissue

Hepatocytes were plated on cover slips 24 hours prior to experimentation. Prior to the experiment, the cells were rinsed with 1X PBS to remove excess FBS and media. The cells were then fixed using 3.5% formaldehyde in 1X PBS for 5 minutes, air-dried, and rinsed twice with wash buffer (1% BSA, 0.05% sodium azide, 0.02% saponin in 1X PBS). Cells were incubated for ½ hour in 37°C at (1:200) concentration for primary antibody and a (1:50) dilution of the secondary antibody. Two rinses with wash buffer between antibody incubations were conducted. Lastly, cells were rinsed twice with wash buffer and twice with 1X PBS, plated onto glass slide and view immediately using confocal microscopy (Nikon). Liver tissues were sent out for sectioned to Cureline biopathology laboratories. The slides with the mounted tissue were fixed with 100% acetone for 15 min in 20°C, followed by 30-minute room temperature incubation in permeabilizing buffer (5% BSA, 0.4% triton-X in PBS). Primary and secondary antibody concentrations were same as previously mentioned. Their incubation; however was done at room temperature for one hour. Cover slips were mounted on top of tissue sections after hybridizing was performed.

Small interfering RNA

Huh7 and primary hepactocytes were transfected with HCV genotypes 1b and 2a, respectively. At time points of three or twenty-four hours, the cells were treated with Nox1 and Nox4 small interfering RNA (siRNA). Nox1 siRNA pool contained sequences, GGUUAGGGCUGAAUGUUUU, CUGCCUACAUACAGCUAUU, GACAAAUACUACUACACAA, and UGAGAAAGCAAUUGGAUCA. Nox4 siRNA single sequence is GAUCACAGCCUCUACAUAU (Dharmacon). For the experiments, 2μg of Nox1, Nox4, or non-targeting siRNA in 1X PBS was added to reduced serum in the media (OPTI-MEM, Invitrogen). In a separate tube, OPTI-MEM was incubated with 1% of a cationic lipid (RNAiMAX, Invitrogen). The positively charged lipid guides the siRNA to the plasma membrane of the hepatocytes facilitating the interaction between the siRNA and the negatively charge plasma membrane. Both solutions were combined and incubated at room temperature for 20 minutes. After the incubation, antibiotic-free media was added and cells were cultured in the media. We found that 72 hours and 96 hours is optimal for silencing of Nox1 and Nox4. Cells treated with the siRNA were used for western blotting, RT-qPCR, and Nox activity assay.

Oxidation of 2'7'-dichlorofluorescein (DCF)

Cells were plated on glass bottom dishes the day before experiment or treated with siRNA 72 or 96 hours before experiment. Cells were rinsed with 1X PBS, and new media was added. A final concentration of 10μM *2'7'-dichlorodihydrofluorescein diacetate* (DCFH-DA) was added to appropriate wells and incubated for ½ hour in 37°C. After incubation, wells were rinsed twice with 1X PBS and immediately imaged using confocal microscopy (Nikon). DCF, the oxidation product of DCFH-DA, was detectable using laser excitation at 543nm and emission at 590/50nm. For inhibition of flavoproteins, cells were incubated with diphenyliodonium (DPI) for an hour before

DCFH-DA incubation. The media was changed and cells were rinsed once with 1X PBS before adding 10μM DPI.

Superoxide detection by high performance liquid chromatography (HPLC)

Dihydroethidine (DHE) can easily pass through cellular plasma membranes and react with superoxide or any other ROS. The product of superoxide and DHE is 2-hydroxyethidium, which is detectable by fluorescence emission at 595nm and A_{290nm}. For this experiment, hepatocytes were prepped as previously described. Collected samples were processed as described in Zielonka *et al* (51). Cells were incubated with a 10μM DHE for about ½ hour and collected using 1ml Dulbecco's phosphate buffered saline (DPBS). To lyse the cell pellets they were re-suspended about 15 times in 0.1% triton-X DPBS solution. Once the cells were lysed, 100μl was transferred to a chilled microcentrifuge tube containing 100μl of 0.2M $HClO_4$ in methanol and incubated on ice for two hours. Mixtures were centrifuged for 30 minutes in 4ºC at 20,000 x g. The supernatant was then transferred to a chilled microcentrifuge tube containing 1M phosphate buffer and centrifuged at 20,000 x g for 15minutes in 4ºC. HPLC amber vials with conical insert were filled with 200μl of the prepared samples and placed on the auto injector for analysis. The elution solvents used were 0.1% tetraflouracetic Acid (TFA) in acetonitrile with 0.1% TFA in H_2O. The UV and fluorescence spectra were captured at A_{290nm} and at 510nm excitation and 595nm emission, respectively, using Perkin Elmer HPLC 200 series system.

Hydrogen peroxide assay

Due to the unavailability of intracellular H_2O_2 detection assays, we used horseradish peroxidase (HRP) reduction with H_2O_2 and *homovanillic acid* for

extracellular peroxide detection method (30). Cultured cells with the appropriate experimental treatments were kept at 37ºC. Upon experimentation, cells were rinsed with 1X PBS and Krebs–Ringer-Phosphate Hepes buffered saline (KRPH) [1.44M NaCl, 0.085M Na_2HPO_4, 0.014M $Na_2H_2PO_4$, 0.05M KCl, 1.3mM $CaCl_2$, 1mM $MgSO_4$, 5mM glucose and 10mM Hepes free acid pH 7.4] was added to the cells. If catalase treatments were used in the experiments, it was added first then followed by a 100μM of *Homovanillic acid* and 5 U/ml of HRP. The plates were wrapped in foil and placed at 37ºC for 15 minutes. The HRP fluorescence was captured by 321nm excitation and 421nm emission. Protein analysis of the cell samples was done using BCA reagents

Superoxide detection by nitro blue tetrazolium (NBT) Assay

Cells were cultured as mentioned previously. On the day of experiment, media was discarded and KRPH was added to the cells with a 0.25μM final concentration of NBT and plates were wrapped in foil and set for a 30 minute incubation at 37ºC. The reaction was terminated after 5-minutes by the addition of 7.4% formaldehyde. Reaction was discarded and wells were rinsed twice with 2X PBS. To quantify the NBT we dissolved it using 2M KOH and DMSO. First 1X PBS was discarded and cells were rinsed with methanol two times to removed un-dissolved NBT. The wells were air-dried and then 2 M KOH was added followed by 100% DMSO was added immediately. The plate was shaken and swirled around to completely dissolve the NBT. The absorbance was measured at 630nm using the spectrophotometer. Protein analysis of each sample was done using BCA reagents.

Statistics

Data was analyzed using Student's t test or one-way analysis of variance using SigmaStat 3.1 (Jandel Scientific). A p-value ≤ 0.05 was considered significant. Data are represented as mean ± standard error of the mean (SEM) of several independent experiments.

Chapter 3: Results

1) Analysis of NAD(P)H oxidases gene expression in cellular models of HCV

qRT-PCR analysis of all seven mRNA transcripts of Nox enzymes revealed that Nox1, Nox4 and Doux2 mRNA levels were elevated 48 and 72 hours post-HCV exposure. At 72 hours Nox4 mRNA level was more than 7 times that of in the mock-transfected control cells. A time course analysis demonstrated that Nox1 and Nox4 were persistently elevated at least to day 17 and 18 (Figure 1 D-F). Doux2 mRNA showed only a transient increase; therefore we focused more on Nox1 and Nox4 (Figure 1 G).

Once the mRNA of Nox1 and Nox4 were demonstrated to be elevated in human hepatoma cells producing HCV particles we further investigated the protein content. Western blotting clearly showed that Nox4 and Nox1 proteins are increased in hepatocytes transfected and infected with HCV genotype 2a (JFH1) (Figure 2 A and B). Liver tissues HCV infected and non-infected were also analyzed and showed even more dramatic increases in Nox1 and Nox4 proteins (Figure 2C).

We further investigated the Nox expression patterns that were observed in hepatocytes with another HCV genotype, using CG1B strain of genotype 1b. Along with CG1b, the primary hepatocytes were also transfected with an empty vector and NS5B-mutated CG1b GND as controls. Genotype (1b) was successfully transfected into telomerase-reconstituted primary hepatocytes (Figure 4A and B), which was demonstrated by the detection of HCV RNAs by qRT-PCR and core proteins by confocal microscopy. After establishing a model for genotype 1b HCV replication, Nox1 and

Nox4 protein contents were examined (Figure 4C). Nox1 and Nox4 proteins increased in hepatocytes with HCV (Figure 6).

2) Determination of NAD(P)H oxidase1 and 4 cellular location

Biochemical sub-cellular fractionations were performed to determine the location of the NOXes. Hepatocytes expressing genotypes 1b (CG1b) and 2a (JFH1) showed NOX4 in the cytoplasmic (C) and nuclear (N) fractions. NOX1 protein was found predominantly in the cytoplasm (Figure 5A-C). Endoplasmic reticulum protein calnexin, inter-membrane protein pan-cadherin, and GAPDH confirmed the cytoplasmic fraction while Lamin A/C and HDAC1 served as nuclear markers. In the fractionation done using primary hepatocytes with HCV-genotype 1b (CG1b) the post-nuclear pellet was included in the western blot analysis. The Nox4 content in the post-nuclear fraction (PN) was increased dramatically with the presence of the virus. Lamin A/C in the PN fraction indicated that the post-nuclear content contained the nuclear membranes.

Immunofluorescence analysis confirmed the results of the sub-cellular fractionations. Nox1 protein, again, was predominantly located in the cytoplasm in cells with CG1b and JFH1, and in liver tissues infected with HCV (Figure 3A-B and Figure 6A-B). Lamin A/C was used as a nuclear marker in the cells treated with HCV genotype CG1b and the liver tissues. Nox4 proteins were found in the cytoplasm and nucleus, showing colocalization with lamin A/C in hepatocytes with genotypes CG1b and in the liver tissues. Propydium Iodide (PI) also showed a complete overlay with Nox4 antibodies in cells transfected with JFH1. The Nox4 location in the JFH cells was further investigated by confocal microscopy using ER marker calnexin and nuclear envelope marker lamin A/C at 48 and 96 hours (Figure 7). The results supported our previous findings that Nox4 resided in cytoplasm and nucleus of cells infected with JFH1. At 96

hours there was a complete overlay of lamin A/C fluorescence and Nox4 antibody, and the same overlay pattern was also seen in cells using PI as the nuclear marker. Overall, the same pattern observed in the subcellular fractionations was shown in the immunofluorescence analysis. Therefore, Nox1 protein was induced by HCV and mainly located in the cytoplasm while the Nox4 proteins were seen in the cytoplasmic, peri-nuclear, and nuclear regions.

3) Assessment of NAD(P)H oxidase enzyme activity

Once the location of the induced Nox proteins was determined, their activity was examined. The first step in determining Nox enzymatic activity was to assess the overall cellular redox state, using H_2-dichlorofluorescein diacetate (DCFH-DA) in mock and JFH1 transfected cells. When there are cellular oxidation or redox modifications in the presence of DCFH the molecule will in turn also get deacetylated and oxidized, become a fluorophore (9). Therefore, the detection of DCF fluorescence serves as an indication that there is an increased oxidation, although it does not identify what type of reactive species is present. Increased fluorescence of DCF was observed in the cells with JFH1 virus compared to mock transfected cells. Adding 10mM H_2O_2 to mock-transfected cells also induced DCF fluorescence and served as a positive control. When cells with HCV-JFH1 were treated with dipheny iodonium (DPI), a flavoprotein inhibitor, the DCF fluorescence decreased significantly (Figure 8).

These results of DCF experiments were supported by an increased detection of ethidium (E+) in the hepatocytes incubated with DHE (Figure 10A). Cells with the siRNA treatments showed a significant decrease in E+ compared to control cells that were

treated with non-targeting siRNA (Figure 10C). The elevation of ethidium in cells transfected with JFH1 viral RNA only informs us of an increase of oxidation in these hepatocytes, and not the reactive species involved. The precise mechanism of the convergence of DHE to E+ is only known to involve some hydride acceptance. Detection of 2-OH-E+ is the marker used to indirectly attempt to quantify the presence of superoxide (37, 51). Therefore, we investigated the specific type of oxygen radicals that increased the overall state of oxidation by measuring H_2O_2 and superoxide anion.

NBT assay was first used for extracellular detection of superoxide anion. The NBT peptide gets reduced by superoxide, changes color and aggregates around plasma membrane. The aggregates of reduced NBT are called formazans (11). SOD was able to decrease the NBT reduction as expected (Figure 9A) but JFH1 did not significantly increase the NBT reduction. The lack of a significant increase in extracellular superoxide is consistent with the lack of increases in Nox1 and Nox4 in the cell periphery in figures 3 and 7.

Control and JFH1 transfected cells were next analyzed for extracellular H_2O_2 content. As expected, when catalase was added to the reaction, the level of hydrogen peroxide of mock transfected cells was decreased by 35.4%. Our results indicate that there is a significant increase in hydrogen peroxide content in JFH1 transfected hepatocytes at 72 and 96 hours post transfection (Figure 9C only 96 hour data). To further assess the role of Nox1 and Nox4, JFH1 transfected and mock-transfected cells were treated with Nox1 and Nox4 siRNAs and analyzed again for H_2O_2. Nox1 and Nox4 siRNAs decreased Nox1 and Nox4 proteins by 42.1% and 68.8%, respectively. The peroxide assay demonstrated a significant decrease of about 48.4% in hydrogen

peroxide in cells treat with Nox4 siRNA with a p-value of 0.002 (Figure 9C) and a 23.4% decrease with Nox1 siRNA.

HPLC analysis of the 2-OH-E+ content indicated that hepatocytes transfected with HCV JFH1 RNA have an elevated 2-OH-E+ content (Figure 10B, P = 0.007). Huh7 cells stimulated with menadione also showed an increase in 2-OH-E+ (Figure 10D), which was enhanced by adding DDC (spell out the first time), an inhibitor of SOD. The data suggest that there is a substantial competition between SOD and the probe for superoxide anion in these cells. Experiments are currently underway to determine whether 2-OH-E+, which increased with JFH1 in figure 10B might also increase further with DDC and can be decreased with siRNAs to Nox1 and Nox4.

Huh7 cells over-expressing Nox1/Nox4 genes were also generated and were analyzed for Nox protein content to ensure that the cells are expressing these proteins (Figure 11A). Nox1 and Nox4 protein levels were increased in these cells, as expected. Huh7 cells stably transfected with Nox4 cDNAs demonstrated an elevated level of hydrogen peroxide compared to control Huh7 cells transfected with the empty plasmid (pCDEF) alone (Figure 11B, P = 0.001). The Nox4 cells also showed increased level of superoxide intracellularly (Figure 11C). In contrast, Nox1 over-expressing cells did not show any significant increase in H_2O_2 or superoxide generation; the data suggests that in the absence of HCV, some regulatory protein(s) necessary for Nox1 activity is/are either not present at high enough levels or not functional to support NOX1 activity.

4) Nuclear detection of nitrotyrosine in HCV-transfected hepatocytes

3-Nitrotyrosine is generated when $O_2^{\bullet-}$ and $NO^{\cdot}$ react to produce peroxynitrite, which in turns leads to the nitration of tyrosine residues in proteins. The hydroxyl group serine and threonine are also candidates for nitration (16). The detection of 3-nitrotyrosine is a well-known indicator of the presence of the incredibly fast reaction between superoxide anion and $NO^{\cdot}$. As shown in Figure 12, we found increased level of 3-nitrotyrosine in JFH1 transfected cells compared to mock transfected cells. There was also perinuclear and nuclear detection of 3-nitrotyrosine in JFH1 transfected hepatocytes. A co-staining with propydium iodine confirmed the nuclear location of 3-nitrotyrosine in hepatocytes 96 hours post JFH1 transfection.

Chapter 4: Discussion

Oxidative stress in HCV infection has been described quite extensively in the literature but the source for ROS has not been clearly demonstrated in the context of complete HCV replication. Previously, Moriya *et al* proposed that the mitochondria generates ROS, which oxidizes the DNA in HCV infections (34). The work that yielded these conclusions contributed significantly to the field, but the experimental models used were limited. The experimental models used were hepatocytes and transgenic mice over-expressing core proteins; therefore, these models do not simulate a natural course of HCV infection in hepatocytes (32, 35). Recent developments of virus producing JFH1 or the CG1b systems present valuable tools for studying the source of ROS during HCV infection. Our JFH1 transfected hepatocytes have a high positive and negative sense genome titer and generate infectious virus particles; thus, making the microenvironment of these hepatocytes more similar to those of HCV infected individuals (Figure 1A-C). Obtaining an infectious cell line for culturing genotype 1b (CG1b) has been difficult in the field; however we were able to mimic genotype 1b infection using telomerase reconstructed fetal hepatocytes as previously shown with Huh7 cells (50). The CG1b ribozyme system is different because DNA is used rather than viral RNA; however CG1b also produces virus particles; therefore, this model is a promising system for studying genotype 1b infection without additional chemical and genetic manipulations. After establishing reasonable working hepatocyte models, the transcripts for all seven Nox were analyzed (Figure 4A). In this work we demonstrated that Nox1, Nox4 and Duox2 gene expression is elevated in human hepatocytes producing infectious hepatitis C virus particles. Nox1 and Nox4 were persistently elevated for at least 30 days post viral RNA transfection (Figure 1D-F; also, data not shown) whereas Duox2 did not show a

consistent induction at later time points (Figure 1G). The western blot analysis of the cell lysate of treated hepatocytes and liver tissues further showed increased levels of these proteins in JFH1 transfected cells. Furthermore, the proteins were also significantly increased in the liver tissue from an HCV infected person (Figure 2A-C).

Nox1 and its regulatory proteins (NOXA 1 and NOXO 1) aggregate at the plasma membrane upon activation, like Nox2 (26,48). The location of NOX4 proteins has been a bit controversial (24). In this study, the subcellular fractionation and immunofluorescence analysis identified Nox1 and Nox4 in the cytoplasm and Nox4 in the nuclear periphery and nucleus of hepatocytes (Figures 5 & 6). Nox1 location was as expected. Interestingly, Nox4 is nuclear; which clearly indicates that it is a possible source of superoxide close to the host DNA. Location of the oxidases was confirmed through multiple experiments using immunofluorescence with confocal microscopy and sub-cellular fractionations using two different protocols (Figure 7). Identifying Nox4 in the nucleus and perinuclear regions generates questions as to where in the nucleus Nox4 is located. Indeed, Nox4 enzymes are membrane bound and contain transmembrane domains. The nucleoplasm is generally considered membrane free. However; intranuclear membrane structures (cisternae) that are are connected to the nuclear envelope and/or the nucleolus have been reported (19). The ER membrane is also contiguous with the nuclear membrane. It is therefore possible that Nox4 resides within the inner or outer nuclear membrane or in the intranuclear cisternae. If Nox4 is responsible for peroxynitrite dependent DNA damage, it would be expected to be located on the inner nuclear membrane with the active site facing the nucleoplasm. Detailed analysis of the subcellular location of Nox4 by electron microscopy and nuclear membrane isolation is on the way.

To evaluate the activity of Nox proteins, we first looked at the overall oxidation state of JFH1 transfected hepatocytes with DCF. Figure 8 clearly demonstrates the increase in DCF fluorescence. DPI decreased the DCF fluorescence dramatically. This told us that the oxidation observed was due to a flavoprotein, possibly NOXes. The fluorescence detection of ethidium confirmed the increased oxidation in JFH1 transfected human hepatocytes (Figure 10A). Our next step in identifying the type of ROS that is causing increased oxidation was to look at the intra- and extracellular superoxide content. Extracellular superoxide was not increased with JFH1 (Figure 9A). Indeed, the superoxide generated from a protein in the nucleus will not be detected extracellularly. However, we clearly detected a much higher H_2O_2 in cells transfected with JFH1 genotype at 72 and 96 hours post transfection. The Nox siRNA studies (Figure 9C) showed a decrease in H_2O_2 with Nox4 and less substantially, with Nox1 siRNAs.

Recently, Dikalov *et al.* suggested that Nox4 generates hydrogen peroxide rather than superoxide (7). Their proposed mechanism is that the dismutation of superoxide occurs extremely fast. However, we clearly saw that the intracellular concentration of superoxide using HPLC was higher in JFH1 transfected hepatocytes. As a positive control, the Huh7 cells were treated with menadione, which caused an increase in the 2-OH-E+ signal compared to control cells (Figure 10D). We also inhibited SOD using DDC and this increased the 2-OH-E+ signal.

Once Nox1/4 activities were assessed, we determined the presence and location of peroxynitrite's "footprint" 3-nitrotyrosine. The immunofluorescence experiments, repeated numerous times, showed that JFH1 transfected hepatocytes have increased levels of 3-nitrotyrosine compared to mock cells (Figure 12). These results agree with

the previous work done by others using liver tissues sections, which clearly demonstrate that patients with HCV infections have an increase of 3-nitrotyrosine (14). The results also indicated that in HCV infected hepatocytes, the nuclear proteins were nitrated. As mentioned before nitration of tyrosine and other residues are induced by peroxynitrite, the product of $NO^{\cdot}$ reacting with superoxide. The presence of peroxynitrite in the nucleus strongly suggest that peroxynitrite-dependent DNA damage will occur, as previously shown by Machida *et al* (32). Preliminary DNA damage experiments done in our laboratory suggest that there is more DNA oxidation in JFH1 transfected hepatocytes compared to mock transfected controls (unpublished findings). Future studies will examine DNA damage and mutations caused by the nuclear ROS, and the role of Nox1 and Nox4 in the chromosomal aberrations in the induction of HCC. ROS generated by Nox enzymes have been hypothesized to play a role in intracellular signaling (26). Also, Nox1 and Nox4 derived ROS has been implicated in the premature senescence of human umbilical vein endothelial cells (HUEVCs) that are growth arrested in the S phase of the cell cycle (38). Therefore, Nox enzymes are likely to participate in HCV-induced pathogenesis by multiple mechanisms, including DNA damage and modulation of host gene expression by nuclear generation of ROS/RNS by Nox4.

Oxidative stress in hepatitis C patients has been suggested as a major cause of liver pathogenesis. Our discoveries provide new insight into possible mechanisms of HCV pathogenesis. Previously, TGF-β, TNF-α, ROS and RNS have been reported to be induced upon HCV infection but still the mechanism has not been clearly identified (14,15,39). Our discovery of the induction of Nox4 in the nucleus along with 3-nitrotyrosine provides a plausible explanation for increased oxidative stress and DNA damage during HCV infection. Figure 13 presents a potential mechanisms by which Nox

enzymes contribute to HCV-induced hepatocarcinogensis. Future direction of the project will be geared towards understanding the role of hepatocyte Nox proteins in the DNA damage and induction of HCC. A detailed analysis of the subcellular location of Nox4 by electron microscopy and nuclear membrane isolation will also be conducted. Furthermore, future studies will continue to identify direct or indirect pathways that HCV utilizes to activate Nox1 and Nox4 and increases the nuclear localization of Nox4.

References

1. Bahoir, B (2004).NADPH oxidase. Current Opinion in Immunology. 16, 42-47.

2. Cardin, R, Saccoccio, G, Masutti, F, Bellentani, S, Farinati, F, & Tiribelli, C (2001). DNA oxidative damage in leukocytes correlates with the severity of HCV-related liver disease: validation in an open population study. 34, 587-592.

3. Cell Biolabs, Inc. (2009). *OxiSelect Oxidative DNA damage ELISA Kit (8-OHdG Quantitation)*

4. Chen, K., Kirber, M. T., Xiao, H., Yang, Y., & Keaney Jr., J. F. (2008) Regulation of ROS signal transduction by NADPH oxidase 4 localization. Journal of Cell Biology. 181(7), [1129-1139].

5. Cool, R, Merten, E, Theiss, C, & Acker, H (1998). Rac1, and not Rac2, is involved in the regulation of intracellular hydrogen peroxide level in HepG2 cells. Biochemistry Journal, 332, 5-8.

6. Digman JD, Lebovitz RM & Roeder RG. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acid Res., 11, [1475-1489].

7. Dikalov, S, Dikalova, A, Bikineyeva, A, Schmidt, H, Harrison, D, & Griendling, K (2008). Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. Free Radical Biology & Medicine. 45, 1340-1351.

8. Division of Viral Hepatitis, (2009). FAQs for Health Professionals. Retrieved August 3, 2009, from Centers for Disease Control and Prevention Web site: http://www.cdc.gov/hepatitis/HCV/HCVfaq.htm#section2.

9. Edger, A. R, Arriaga, A. E. (2006) Capillary Electophoresis Monitors Enhancement in Subcellular Reactive Oxygen Species Production upon Treatment with Doxorubicin. Chemical Research in Toxicology. 19, [1151-1159].

10. Farinati, F., Cardin, R., Bortolami, M., Burra, P., Russo, F.P., Rugge, M., Guido, M., Sergio, A., & Naccarato, R. (2007). Journal of Viral hepatitis. Hepatitis C virus: from oxygen free radicals to hepatocellular carcinoma. 14, [821-829].

11. Freeman, R. & King, B. (1972) Technique for the performance of the nitro-blue tretrazolium (NBT) test. Technical Methods. [912-914].

12. Fujita, N., Horike, S., Sugimoto, R., Tanaka, H., Iwasa, M., Kobayashi, Y., Hasegawa, K., Ma, N., Kawanishi, S., Adachi, K., & Kaitoa, M. (2007). Hepatic Oxidative DNA damage correlates with iron overload in chronic hepatitis C patients. Free Radical Biology & Medicine. 42, 353-362.

13. Garcia-Mediavilla, M. V., Sanchez-Campos, S., Gonzalez-Perez, P., Gomez-Gonzalo, M., Majano, P. L., Lopez-Cabrera, M., Clemente, G., Garcia-Monzon, C., & Gonzalez-Gallego, J. (2005) Differential contribution of hepatitis C virus NS5A and core proteins to the induction of oxidative and nitrosative stress in human hepatocytes-derived cells. Journal of Hepatology. 43, [606-613].

14. Garcia-Monzon, C., Majano, P. L., Zubia, I., Sanz, P., Apolinario, A., & Moreno-Otero R. (2000) Intrahepatic accumulation of nitrotyrosine in chronic viral

hepatitis is associated with histological severity of liver disease. Journal of hepatology. 32, [331-338].

15. Gonzalez-Amaro, R., Garcia-Monzon, C., Garcia-Buey, L., Moreno-Otero, R., Alonso, J. L., Yague, E., Pivel, J. P., Lopez-Cabrera, M., Fernandez-Ruiz, E., & Sanchez-Madrid, F. (1994) Induction of Tumor Necrosis Factor alpha Production by Human Hepatocytes in Chronic Viral Hepatitis. Journal of Experimental Medicine. 179, [841-848].

16. HalliWell, B., & Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine*.Oxford: Oxford University Press Inc..

17. Hohn, D.C., & Lehrer, R.L. (1974). NADPH Oxidase Deficiency in X-Linked. The Journal of Clinical Investigation. 55, [707-713].

18. Holmgren, A. (2000) Antioxidant Function of Thioredoxin and Glutaredoxin Systems. Antioxidants & Redox signaling. 2, [811-820].

19. Isaac, C., Pollard, J. W., Meier, T. (2001). Intranuclear endoplasmic reticulum induced by Nopp140 mimics the nucleor channel system of human endometrium. Journal of Cell Science. 114, [4256-4264].

20. Janeway, C. (2005). *Immunology the immune system in health and disease*. New York, NY: Garland Science Publishing.

21. Jaurand, M.C. (1997).Mechanisms of Fiber-induced Genotoxicity. Environment Health Prospect. 105, 1073-1084.

22. Joyce, M. A., Walter, K-A., Lamb, S. E., Yeh, M. M., Zhu, L. F., Kneteman, N., Doyle, J. S., Katze, M. G., & Tyrrell, D. L. (2009) HCV Induces Oxidative and ER

stress, and Sensitizes Infected Cells to Apoptosis in SCID.Alb-uPA Mice. PLoS Pathogens. 5(2).

23. Korenaga, M, Wang, T, Li, Y, Showalter, L.A., Chan, T, & Sun, J (2005). Hepatitis C Virus Core Protein Inhibits Mitochondrial Electron Transport and Increases Reactive Oxygen Species (ROS) Production. 280, 37481-37488.

24. Kuroda, J., Nakagawa, K., Yamasaki, T., Nakamura, K., Takeya, R., Kuribayashi, F., Imajoh-Ohmi, S., Igarashi, K., Shibata, Y., Sueishi, K., & Sumimoto, H. (2005) The superoxide-producing NAD(P)H oxidase Nox4 in the nucleus of huan vascular endothelial cells. Genes to Cells. 10, [1139-1151].

25. Lambeth, J.D. (2000).Regulation of the Phagocyte Respiratory Burst Oxidase by Protein Interactions. Journal of Biochemistry and Molecular Biology. 33, 427-439.

26. Lambeth, J. D. Nox/Duox family of nicotinamide adenine dinucleotide (phosphate) oxidases. Current Opinion in Hematology. 9, [11-17].

27. Li, J.M., & Shah, A.M. (2002). Intracellular Localization and Preassembly of the NDAPH Oxidase Complex in Cultured Endothelial Cells. The Journcal of Biological Chemistry. 277, [19952-19960].

28. Li, J.M., & Shah, A.M. (2003). ROS Generation by Nonphagocytic NADPH Oxidase: Potential Relevance in Diabetic Nephropathy. Journal of the American Society of Nephrology. 14, [S221-S226].

29. Li, S., Tabar, S.S., Malec, V., Eul, B.G., Klepetko, W., Weissmann, N., Grimminger. F., Seeger, W., Rose, F., & Hanze, J. (2008). NOX4 Regulates ROS Levels under Normoxic and Hypoxic Conditions, Triggers Proliferation, and

Inhibits Apoptosis in Pulmonary Artery Adventitial Fibroblasts. Antioxidants and Redox Signaling.10, [1-9].

30. Liu, R. M., Liu, Y., Forman, H. J., Olman, M., Tarpey, M. M. (2004). Glutathione regulates transforming growth factor-stimulated collagen production in fibroblasts. American Journal Lung Cellular and Molecular Physiology. 282, [L121-L128].

31. Lu, L., Wei, L., Peng, G., Mu, Y., Wu, K., Kang, L., Xioahong, Y., Zhu, Y., & Wu, J. (2008). NS3 protein of hepatitis C virus regulates cyclooxygenase-2 expression through multiple signaling pathways. Virology. 371, [61-70].

32. Machida, K, Cheng, K.T.H., Lai, C.K., Jeng, K.S., Sung, V.M.H., & Lai, M.M.C. (2006). Hepatitis C Virues Triggers Mitochondrial Permeability Transition with Production of Reactive Oxygen Species, Leading to DNA Damage and STAT3 Activation. 80, 7199-7207.

33. Majano, P. L., Garcia-Monzon, C., Lopez-Cabrera, M., Lara-Pezzi, E., Fernandez-Ruiz, E., Gargia-Iglesias, C., Borque, M. J., & Moreno-Otero, R. (1998) Inducible Nitric Oxide Synthase Expression in Chronic Viral Hepatitis. The American Society for Clinical Investigation. 101(7), [1343-1352].

34. Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Mastsuura, Y., Miyamura, T., Koike, K. (1997). Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. Journal of General Virology. 78, [1521-1531].

35. Okuda, M, Li, K, Beard, M.R., Showalter, L.A., Scholle, F., & Lemon, S.M. (2002). Mitochondrial Injury, Oxidative Stress, and Antioxidant Gene Expression Are Induced by Hepatitis C Virus Core Protein. 122, 366-375.

36. Reichheld, J.P., Khafif, M., Riondet, C., Droux, M., Bonnard, G., & Meyer, Y. (2007) Inactivation of Thioredoxin Reductases Reveals a Complex Interplay between Thioredoxin and Glutathione Pathways in Arabidopsis Development. The Plant Cell. 19, [1851-1865].

37. Robinson, K.M., Janes, M.S., Pehar, M, Monette, J.S., Ross, M.F., & Hagen, T.M. (2006). Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. 103, 15038-15043.

38. Schilder, Y. D. C., Heiss, E. H., Schachner, D., Ziegler, J., Reznicek, G., Sorescu, D. & Dirsch, V. (2009) NADPH oxidases 1 and 4 mediate cellular senescence induced by resveratrol in human endothelial cells. Free Radical Biology & Medicine. 46, [1598-1606].

39. Schweyer, S., Mihn, S., Radzum, S., Hartmann, H., Fayyazi, A. (2000) Liver infiltrating T lymphocytes express interferon gamma and inducible nitric oxide synthase in chronic hepatitis C virus infection. GUT. 46, [255-259].

40. Sekoguchi, S., Nakajima, T., Moriguchi, M., Jo, Masayasu, Nishikawa, T., Katagishi, T., Kimura, H., Minami, M., Itoh, Y., Kagawa, K., Tani, Y., & Okanoue, T. (2007). Role of cell-cycle turnover and oxidative stress in telomere shortening and cellular senescence in patients with chronic hepatitis C. Hepatology. 22, [182-190].

41. Seronello, S., Sheikh, M. Y., Choi, J. (2007) Redox regulation of hepatitis C in non-alcoholic and alcoholic liver. Free Radical Biology & Medicine. 43(6), [869-882]

42. Shatwell, K.P., & Segal, A.W. (1996). NADPH Oxidase. The International Journal of Biochemistry & Cell Biology. 28, 1191-1195.

43. Sheikh, M.Y., Choi, J., Qadri, I., Friedman, J.E., & Sanyal, A.J. (2008). Hepatitis C Virus Infection: Molecular Pathways to Metabolic Syndrome. Hepatology. 47, [2127-2133].

44. Shiose, A., Kuroda, J., Tsuruya, K., Hirai, M., Hirakata, H., Naito, S., Hattori, M., Sakaki, Y., & Sumimoto, H. (2001). A Novel Superoxide-producing NAD(P)H Oxidase in Kidney. The Journal of Biological Chemistry. 276, [1417-1423].

45. Shimoda, R, Nagashima, M, Sakamoto, M, Yamaguchi, N, Hirohashi, S, Yokota, J & Kasai, H (1994). Increased Formation of Oxidative DNA Damage, 8-Hydroxydeoxyguanosine. Cancer Research. 54, 3171-3172.

46. Sumimoto, H, Miyano, K, & Takeya, R (2005). Molecular composition and regulation of the Nox family NAD(P)H oxidases. Biochemical and Biophysical Research Communications. 338, 677-686.

47. Tan, S., Pause, A, & Sonenberg, N (2002). Hepatitis C Therapeutics: current status emerging strategies. Nature Reviews. 1, 867-881.

48. Ueyama, T., Geiszt, M., and Leto, T. L. (2006) Involvement of Rac1 in Activation of Multicomponent Nox1-and Nox3-Based NADPH Oxidases. Molecular and Cellular Biology. 26(6), [2161-2174].

49. Worman, H.J. (2002). Hepatitis C. Retrieved July 24, 2009, Web site: http://www.cumc.columbia.edu/dept/gi/hepC.html.

50. Wege, H., Le, H. T., Chui, M. S., Liu, L., Wu, J., Giri, R., Malhi, H., Sappal, B. S., Kumaran, V., Gupta, S., & Zern, M. A. (2003) Gastroenterology. 124, [432-444].

51. Zielonka, J., Vasquez-Vivar, J., & Kalyanaraman, B. (2008). Detection of 2-Hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. Nature Protocols. 3, 8-21.

52. (02-Apr-2009). Entrez Gene: SOD2. Retrieved April 13, 2009, from NCBI Web site: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=6648

53. (02-Apr-2009). Entrez Gene: SOD3. Retrieved April 13, 2009, from NCBI Web site: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=6649