Review

Mitochondrial dysfunction in hepatitis C virus infection

C. Piccoli, R. Scrima, A. D'Aprile, M. Ripoli, L. Lecce, D. Boffoli, N. Capitanio *

Department of Biomedical Science, University of Foggia, viale L. Pinto OO.RR. 71100 Foggia, Italy

Abstract

The mechanisms of liver injury in chronic hepatitis C virus (HCV) infection are poorly understood though HCV induces a state of hepatic oxidative stress that is more pronounced than that present in many other inflammatory diseases. This mini-review will focus on recent findings revealing an unexpected role of mitochondria in providing a central role in the innate immunity and in addition will illustrate the application of stably transfected human-derived cell lines, inducibly expressing the entire HCV open reading frame for in vitro studies on mitochondria. Results obtained by a comparative analysis of the respiratory chain complexes activities along with mitochondrial morpho-functional confocal microscopy imaging show a detrimental effect of HCV proteins on the cell oxidative metabolism with specific inhibition of complex I activity, decrease of mt$\Delta\Psi$, increased production of reactive oxygen species. A possible de-regulation of calcium recycling between the endoplasmic reticulum and the mitochondrial network is discussed to provide new insights in the pathogenesis of hepatitis C.
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1. Introduction

The hepatitis C virus (HCV) is a significant health problem worldwide. More than 170 million people, or 3% of the world population, are infected with HCV [1]. HCV infection causes acute hepatitis which is naturally cleared in 20–30% of patients. However in 70–80% of cases, HCV persists causing chronic hepatitis [2]. Cycles of hepatocyte death and regeneration occur and activation of stellate cells results in formation of abnormal extracellular matrix. These effects result in progressive fibrosis and carcinogenesis. As a consequence chronic hepatitis can lead to a spectrum of diseases including, fibrosis, cirrhosis and hepatocellular carcinoma [2]. There is no effective vaccine available and current therapies show limited efficacy for many HCV-infected patients [3].

HCV is an enveloped, positive-strand RNA virus and its RNA genome is about 9600 nucleotides in length [4,5]. It carries 5′ and 3′ non coding regions flanking a large open reading frame, which encodes a polyprotein of more than 3000 amino acid residues (Fig. 1). The 5′ NCR contains an internal ribosome entry site that directs the translation of the HCV polyprotein at the level of the endoplasmic reticulum (ER). This polyprotein is cleaved co- and post-translationally by host and viral proteases producing 3 structural proteins (core, E1 and E2) and 7 nonstructural proteins [4,5]. The nonstructural proteins orchestrate viral replication forming a membrane associated replication complex. With the exception of E1 and E2, which face the ER lumen, all the other HCV proteins are mainly exposed to the cytosolic space and are anchored to the ER membrane by specific membrane segments [6].

2. Mitochondria in the innate immunity (MAVS vs. NS3/4a)

Very recently, an unexpected finding has revealed for mitochondria a central role in the innate immunity response of cell to virus infection. Virus infection is known to result in the activation of multiple pathways leading to the expression of genes whose products have inhibitory activity on the viral replication/transcription machinery. A newly discovered protein called MAVS (also named CARDIF,

* Corresponding author. Tel.: +39 0881 711148; fax: +39 0881 714745.
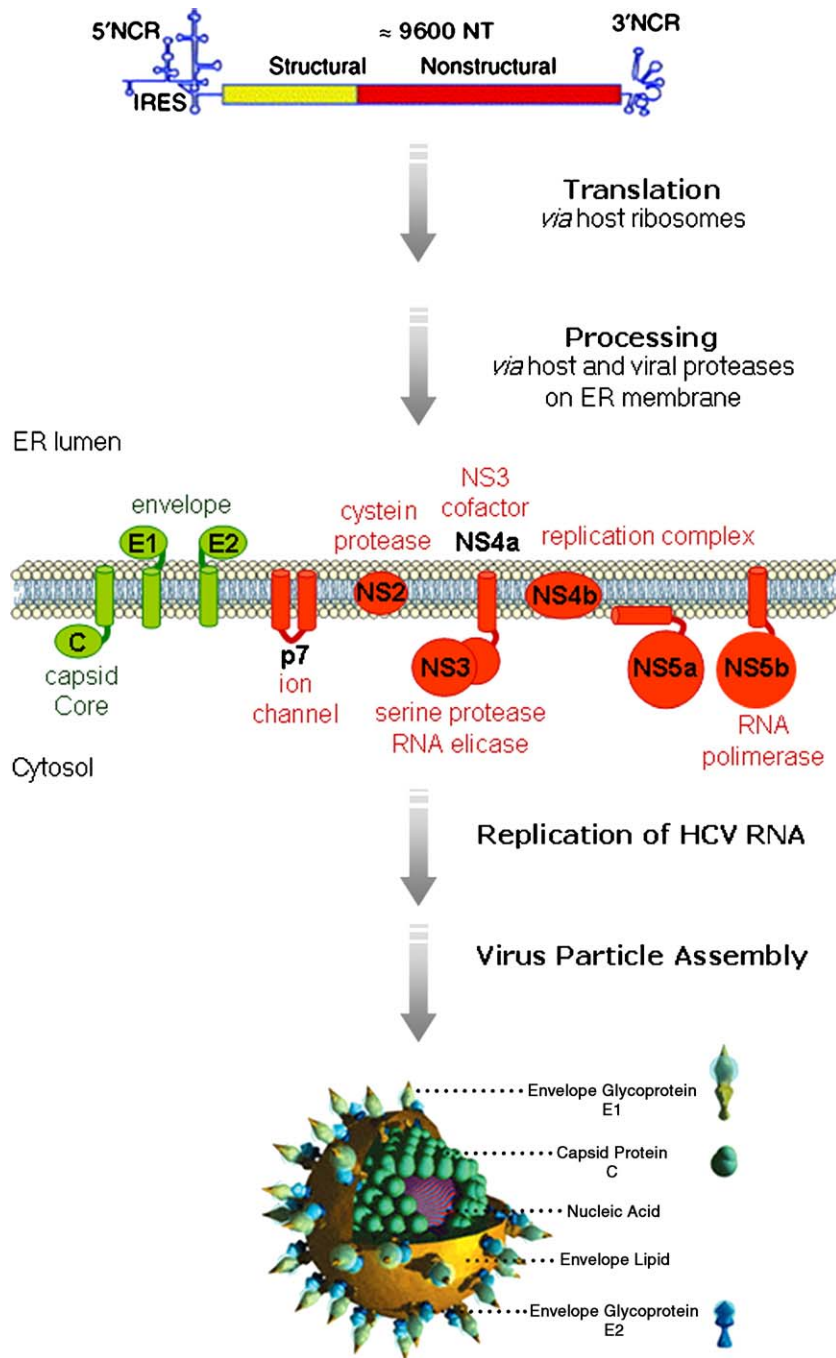 *E-mail address:* n.cap@unifg.it (N. Capitanio).

Fig. 1. Hepatisis C virus (HCV) features. Genome organization and schematic representation of the HCV structural proteins (C, E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) as the proteins associate with the endoplasmic reticulum. The function of the non-structural proteins is also indicated. Another HCV protein named "F" might also be synthesized by a ribosomal frameshift in the core coding sequence. E1 and E2 are N-glycosylated. 5′ and 3′NCR, non coding region; IRES, internal ribosome entry site. Initially, the HCV virus recognizes and is incorporated into human liver cells. The internalized virus then dissociates, liberating the viral RNA genome. The HCV RNA is then translated by the host ribosomes, producing the HCV polypeptide. This polypeptide is subsequently processed, first by host peptidases then by the HCV proteases (NS2 and NS3) into 10 different HCV proteins. The non-structural proteins (NS2–NS5b) are next assembled and localized within the liver cell to form a replication complex, which produces multiple copies of the HCV RNA genome. These RNA copies are then able to re-enter the life cycle, producing more HCV proteins. Eventually, the HCV structural proteins (C, E1 and E2) along with copies of the HCV RNA are packaged as infectious virus particles, released from the liver cell, and are able to infect new cells.

VISA or IPS-1) [7–9] co-ordinates specific pathways required for the activation of IFN-β. This protein is located at the outer mitochondrial membrane where it is anchored by a C-terminal TM helix (Fig. 2). An upstream receptor called RIG-I acts as a sentinel detecting the presence of viral replication intermediates, such as dsRNA. RIG-I binds to dsRNA and interacts with MAVS, the so formed heterocomplex, recruiting different clusters of adapters,
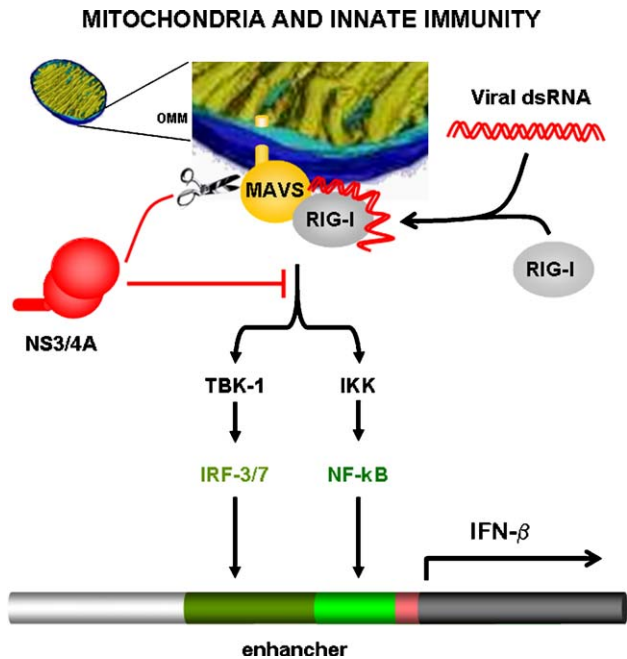
MITOCHONDRIA AND INNATE IMMUNITY

Fig. 2. Schematic drawing illustrating the proposed role of mitochondria in the innate immunity and the strategy used by HCV to evade it. Viral infection results in the activation of multiple pathways. The MAVS (mitochondrial antiviral signalling) protein coordinates upstream events leading to activation of the interferon-β (IFN-β). MAVS (also known as IPS-1, VISA and CARDIF) is localized at the outer mitochondrial membrane where it is anchored by a transmembrane helix. The presence of viral RNA replication products is sensed by the cytosolic protein RIG-I (retinoic acid induced gene I) which contains a RNA helicase domain that binds to dsRNA. RIG-I also contains tandem N-terminal caspase recruitment domains (CARDs) that interact with another CARD domain present in the MAVS protein. Binding of RIG-I complexed to dsRNA to MAVS activates downstream signalling pathways. These include activation of the IKK (inhibitor κB kinase) complex (which regulate NF-κB (nuclear factor κB)) and of the non "canonical" kinase TBK-1 (which regulates IRF3/7 (IFN regulatory factor 3/7)). Activation of these transcription factors and their translocation to the nucleus leads to assembly of a multiprotein enhancer complex, the enhanceosome, which drives expression of IFN-β gene. Importantly, the mitochondrial localization of MAVS is essential for its signalling function, because the removal of the mitochondrial targeting domain of MAVS abolishes its function to induce IFNs. The HCV protein NS3/4A (which is a serine protease) cleaves MAVS at Cys-508, resulting in dislocation of the N-terminal fragment of MAVS from the mitochondria. Point mutation of MAVS at Cys-508 renders MAVS resistant to cleavage by NS3/4A, thus maintaining the ability of MAVS to induce interferons in HCV replicon cells. The physical interaction between the ER-bound NS3/4A and the mitochondria-bound MAVS might be provided by contiguity at localised contact sites of the ER-mitochondria networks or by transient membrane fusion-driven intermembrane protein transfer. Drawn from the evidence reported in Refs. [7–11].

activates at least two protein kinase pathways, that ultimately result in the activation of IRF-3/7 and NF-κB. These transcription factors assemble in an enhansosome driving the expression of IFN-β. Moreover it has been found that one of the nonstructural HCV protein NS 3/4A is able to cleave MAVS (CARDIF) at a specific cysteine residue resulting in its inactivation. This enables HCV to block the MAVS-down-stream signalling pathway and evade the host innate immunity. Mutation of MAVS at that specific

cysteine residue confers resistance of cell to HCV infection [10,11].

Besides this very new immunological role of mitochondria a large body of evidence have been accumulating over the time providing clues for a substantial alteration in the mitochondrial oxidative metabolism although the mechanism has remained elusive [12,13].

3. Cell lines allowing regulated expression of HCV proteins: principles and applications

The lack of an efficient cell culture system, permissive for hepatitis C virus infection and replication, has limited the systematic study of the viral life cycle, pathogenetical mechanisms as well as the development and evaluation of novel antiviral strategies. Infection of primary hepatocytes or transient expression of subgenomic or entire HCV replicons yield only low levels and often poorly reproducible viral replication [4,5]. An alternative approach, more recently developed, involves the generation of cell lines stably expressing viral sequences from chromosomally integrated cDNA constructs [14]. A recent major breakthrough in this direction has been the use of a gene expression system based on regulatory elements of the tetracycline (TET)-resistence operon of *E. coli* (illustrated in Fig. 3A) [15–17]. Inducible cell lines were generated by two successive transfection and selection steps. A founder cell line constitutively expressing the TET-controlled trans-activator was established first, followed by a stable transfection of the cDNA construct, harbouring the HCV genome, under the transcriptional control of a TET-controlled transactivator-dependent promoter. More importantly, steady state HCV gene expression can, not only be switched on and off, but fine-tuned over a broad range by varying the concentration of TET in the culture medium.

Fig. 3B shows the modulation of the HCV protein expression in the human-derived osteosarcoma cell line U-2 OS. Immunoblotting against three of the HCV proteins was undetectable in the non-induced cell line their expression becoming clearly evident following a 48 h withdrawal of TET from the medium. A stationary expression was reached even earlier (data not shown). The expression and intracellular localization of the induced proteins was further verified by confocal microscopy immuno-cytochemistry. The representative images shown in Fig. 3B refer to the structural HCV core protein. It can be seen that the core protein localizes in a web resembling the endoplasmic reticulum. To notice, a more evident spotted structure, is also observed that might indicate the concentration of the HCV proteins in lipid droplets as well documented by immunohistochemical analysis carried out on bioptic reports from infected patients [4,5].

4. Effect of HCV-protein expression on the mitochondrial OXPHOS system

By exploiting this well controlled cell culture system, we studied the effect of the expressed HCV proteins on the mitochondrial OXPHOS system. Differently from other
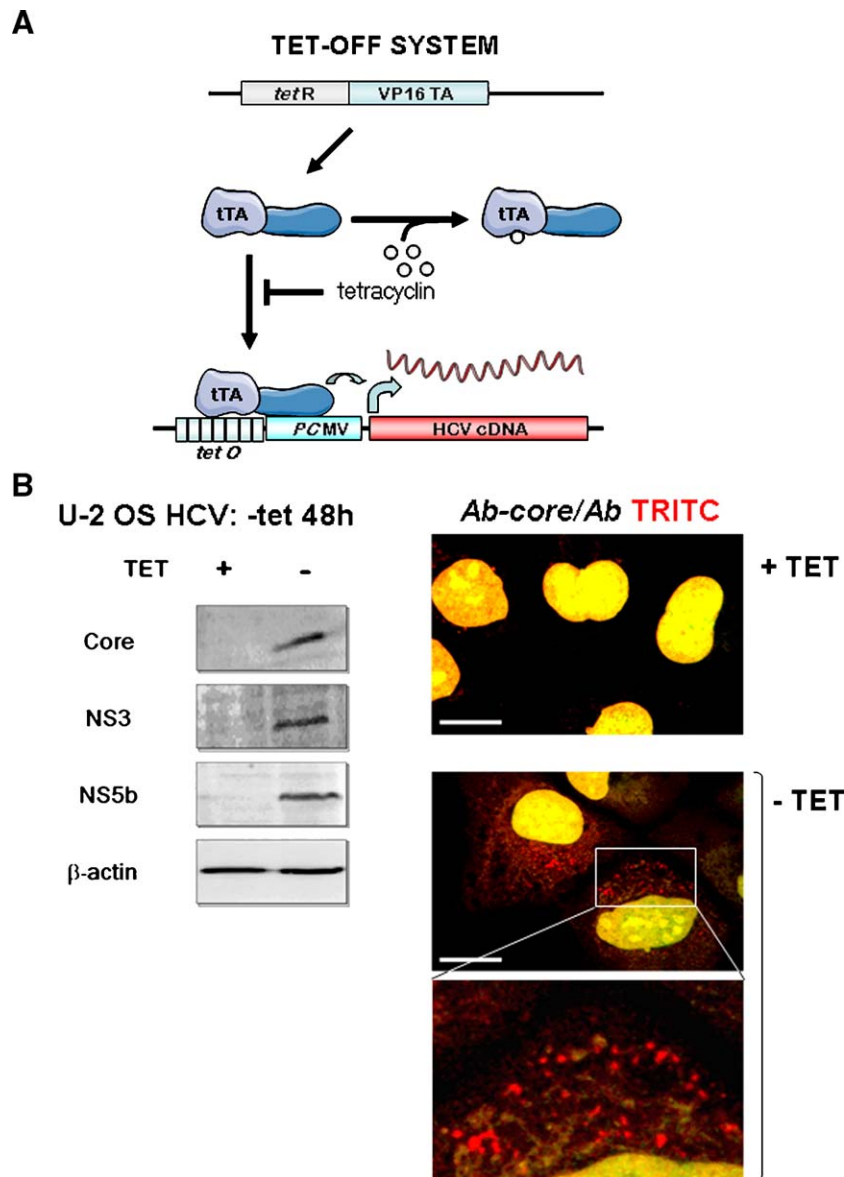
Fig. 3. Regulated HCV-gene expression in U-2 OS-derived cell line. (A) Tetracycline-regulated gene expression system. The system consists of a tetracycline-controlled transactivator (rTA), which is composed of the tetracycline repressor (tet R) fused to the activating domain of VP16 of herpes simplex virus, and of a tTA-dependent promoter, which is composed of a minimal sequence derived from the cytomegalovirus intermediate early promoter (CMV P) combined with heptameric tetracycline operator (tet O) sequences. The tTA-dependent promoter is virtually silent in many cell types in the presence of low concentrations of tetracycline (TET), which prevent the tTA from binding to tet O sequences. In the absence of tetracycline, the tTA binds to the tet O sequences to activate transcription from the minimal promoter. (B) Tightly regulated expression of HCV proteins in U-2 OS cell line. U-2 OS cells were cultured in DMEM supplemented with 10% FBS in the presence of 1 μg/ml tetracycline. Subsequently, in one sample the medium was changed with withdrawal of tetracycline (TET−) and in the other the medium was replaced without omitting tetracycline. After 48 h the cell samples were harvested and assayed. Panel on the left: proteins from cell lysate were separated by 12% SDS-PAGE and analysed by immunoblot with a pool of monoclonal antibodies against core, NS3, NS5b and β-actin. Right panel: immuno-cytochemical detection of HCV-core protein by laser scanning confocal microscopy (LSCM). U-2 OS cells, cultured at low density onto fibronectin coated 35 mm glass bottom dishes as described before, were fixed (4% paraformaldehyde), permeabilised (0.2% Triton X-100), blocked (3% BSA in PBS), and then sequentially incubated with diluted mouse monoclonal anti-core and secondary Rhodamine-conjugated goat anti-mouse IgG. Subsequently cells were treated for nuclear staining with 1 μM Topro-1. Then after cells were examined by a Nikon TE 2000 microscope (images collected using a 60X objective 1.4 NA) coupled to a Radiance 2100 dual laser scanning confocal microscopy system (Biorad). The fluorescence signals of Rhodamine and Topro-1 were monitored sequentially exciting first with the He–Ne laser beam ($\lambda_{ex}=543$ nm) and then with the Ar–Kr laser beam ($\lambda_{ex}=514$ nm) respectively. Magnification of the selected area (indicated by the white frame) is shown for the HCV-induced U-2 OS cell sample. The images are representative of three different preparations. Analogous results were obtained for the immuno-localization of the HCV-NS2 protein. Scale bars: 20 μm.

similar studies, which used transfected cells overexpressing subgenomic HCV constructs, our cell system allows to evaluate the effects caused by the co-ordinated expression of the entire HCV polyprotein thus constituting a better physiological simulation of HCV infection. Of notice, the biological activity of some of the HCV proteins depends on the co-expression of others [4–6]. Fig. 4A shows the results of a respirometric analysis carried out on intact U-2 OS-

derived cell lines. The endogenous respiratory activity was found significantly decreased by a 40% in cells expressing the HCV proteins. Addition of oligomycin strongly reduced the oxygen consumption rates more in the control than in the induced cells so that the relative respiratory control ratio resulted markedly depressed in cells expressing the HCV genome. Incubation of the induced cells with cyclosporine A was ineffective (not shown).

Fig. 4B illustrates the results of a comparative confocal microscopy analysis of the mitochondrial morpho-functional pattern. Using a specific ratiometric dual emitter probe (MitoCapture) it was possible to unveil a profound difference. While in the control cells the almost entire intracellular mitochondrial network was able to generate and maintain relatively high mt$\Delta\Psi$, the HCV-induced cells showed a

functional mitochondrial heterogeneity with many hypo-polarized mitochondria, thus confirming the respiratory measurements. Other specific mitochondrial probes showed that the overall mitochondrial content and architecture was, however, unchanged exhibiting both in the control and in the HCV-induced U-2 OS derived cell lines a well-defined reticular net (not shown).

Fig. 4C shows that measurements of the specific activities of the proton pumping respiratory complexes resulted in inhibition of complex I in the HCV-induced cells to an extent comparable with that observed for the endogenous respiration. Conversely, the activities of complexes III and IV were slightly increased, albeit within the statistical significance, perhaps reflecting some kind of compensatory attempt.

These results show that expression of HCV polyprotein causes an apparent decrease of the OXPHOS coupling efficiency together with an effect on the activity of complex I that manifest on the overall cell respiratory rate.
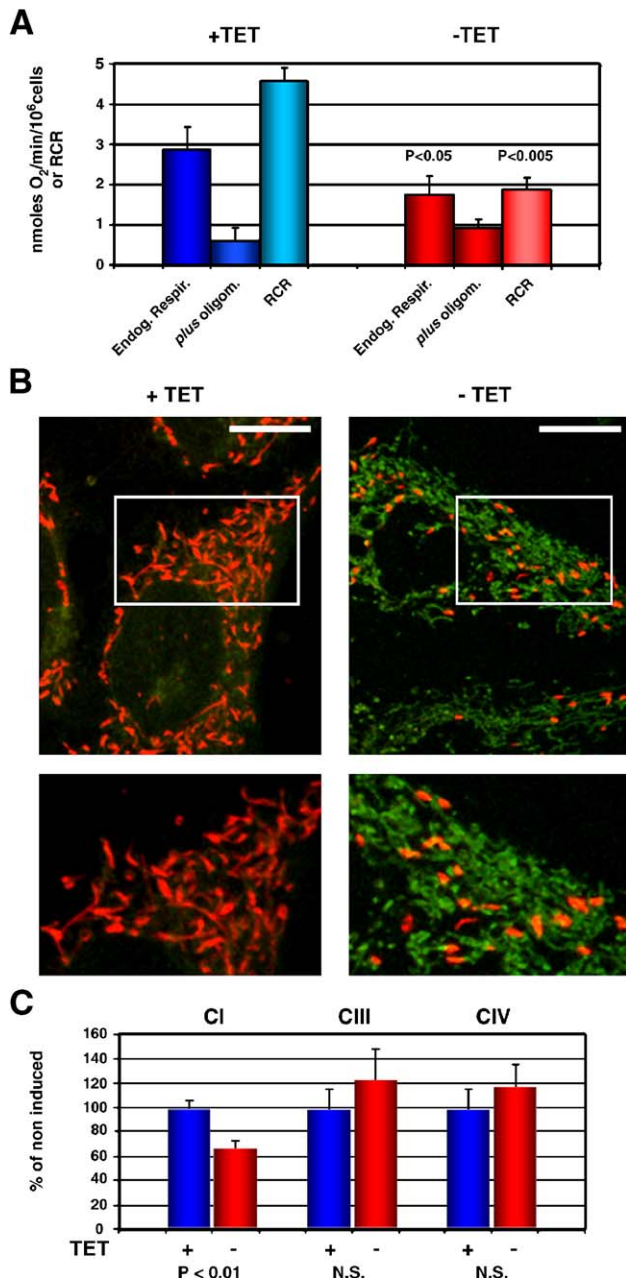

Fig. 4. Functional analysis of HCV protein induction in U-2 OS-derived cell line on the mitochondrial OXPHOS system. (A) Measurements of oxygen consumption in intact cells. $5-7 \times 10^6$ viable cell/ml was assayed polarographycally in 50 mM KPi, 10 mM HEPES, 1 mM EDTA, pH 7.4; after establishment of a stationary endogenous respiratory rate 2 µg/ml of oligomycin was added. Both rates of O_2 consumption were corrected for 2 mM KCN-insensitive respiration. RCR: respiratory control ratio obtained dividing the rates of oxygen consumption attained before and after addition of oligomycin. The values reported are mean (±S.E.M.) of 6 independent preparations. Two tailed Student's t test was applied to evaluate the significance between the rates measured with the non induced (+TET) and induced (−TET) cells. The P value for significant differences is shown. (B) Imaging of $\Delta\Psi$-generating mitochondria by LSCM. Cells were seeded at low density onto fibronectin coated 35 mm glass bottom dishes. After adhesion, living cells were directly incubated for 20 min at 37 °C with MitoCapture (1/1000 dilution). Stained cells were washed with PBS and examined by a Nikon TE 2000 microscope (images collected using a 60X objective 1.4 NA) coupled to a Radiance 2100 dual laser scanning confocal microscopy system (Biorad). The fluorescent signal of the MitoCapture double-emitter probe was examined sequentially, exciting first with the Ar–Kr laser beam (λ_{ex}=488 nm) and then with the He–Ne laser beam (λ_{ex}=543 nm). MitoCapture is a lipophilic cation that accumulates electrophoretically in mitochondria; depending on the extent of the trans-membrane electrical potential the dye accumulates as monomer (green fluorescence emitter) or dimer (red fluorescence emitter, in iper-polarized mitochondria). Magnification of selected areas (indicated by the white frame are shown below each panel. The images are representative of five different preparations. Scale bars: 20 µm. (C) Measurements of the specific activities of NADH-CoQ oxidoreductase (CI), cytochrome c reductase (CIII) and cytochrome c oxidase (CIV). The activities were determined spectrophoto-metrically on mitoplast fraction of ultrasound-treated U-2 OS cells in 10 mM Tris, 1 mg/ml serum albumin, pH 7.4. CI was assayed (in the presence of antimycin A plus KCN) by following the initial rotenone-sensitive rate of NADH oxidation (ΔA at 340 nm) in the presence of UQ$_2$ as electron acceptor; CIII was assayed (in the presence of rotenone plus KCN) by following the initial antimycin A-sensitive rate of cytochrome c reduction (ΔA at 550 nm) in the presence of UQ$_2$ as electron donor; CIV was assayed by following (in the presence of antimycin A) the initial KCN-sensitive rate of cytochrome c oxidation (ΔA at 550 nm) under aerobic conditions. The values reported for the induced cells (TET−) are expressed as percentage of the non-induced (TET+) control cells. Averages (±S.E.M.) of at least 5 different preparations; N.S., not significant.
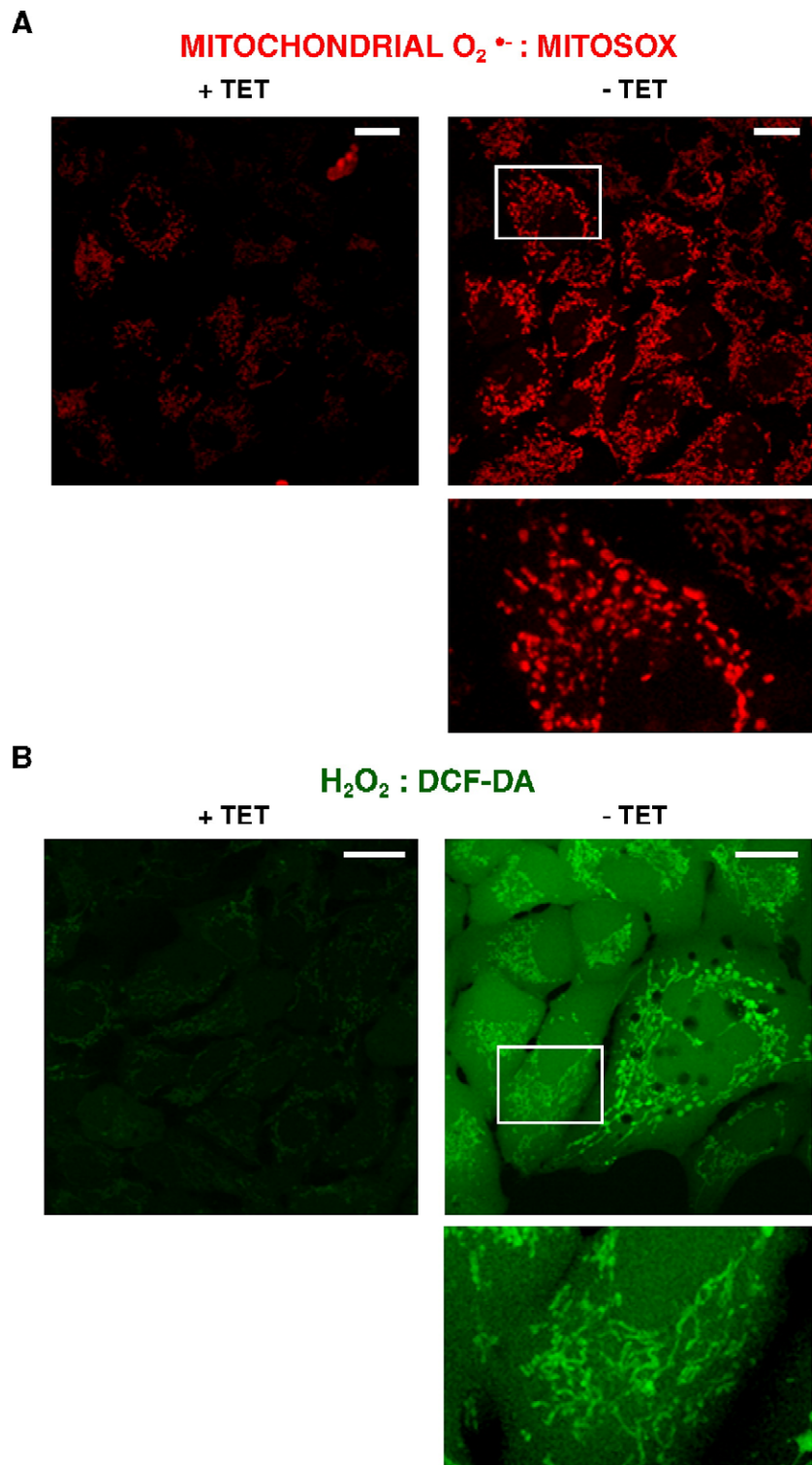
Fig. 5. LSCM analysis of reactive oxygen species (ROS) production in non-induced and HCV-induced U-2 OS-derived cell line. Imaging of intracellular ROS production by the $O_2^{\bullet-}$ and H_2O_2 sensitive probes MitoSOX (A) and DCF (B) respectively. Cells were seeded at low density onto fibronectin coated 35 mm glass bottom dishes. After adhesion, living cells were directly incubated for 20 min at 37 °C with 3 μM MitoSOX or 10 μM dichlorofluorescin-diacetate DCF-DA. Stained cells were washed with PBS and examined as indicated in the legend to Fig. 5. The red fluorescence of MitoSOX, highly selective for detection of superoxide in mitochondria of live cells, was monitored exciting with the He–Ne laser beam (λ_{ex}=543 nm). DCF-DA is a membrane permeant probe which is hydrolised by intracellular esterases and converted to the membrane-impermeant and ROS (mainly H_2O_2)-reacting product DCF. The green fluorescence of oxidised DCF was analysed by exciting the sample with the Ar–Kr laser beam (λ_{ex}=488 nm). Magnification of selected areas (indicated by the white frame are shown below each panel. The images are representative of five different preparations. Scale bars: 20 μm.
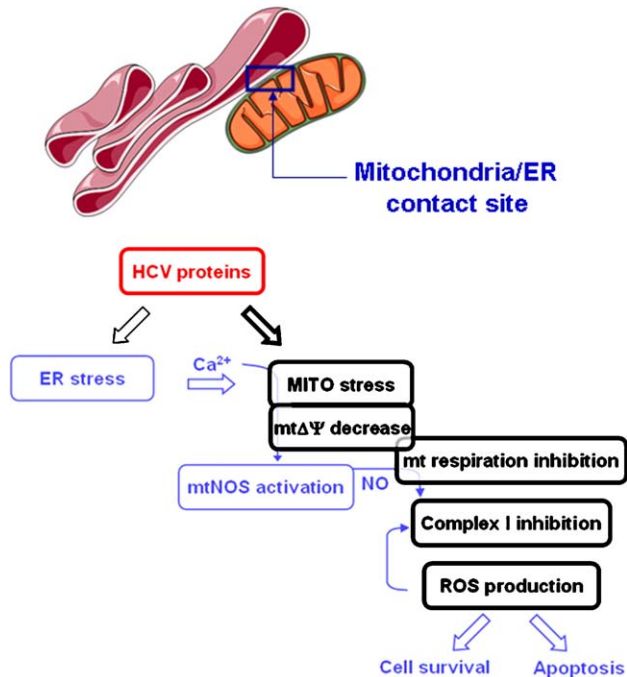
Fig. 6. Schematic working model for the mitochondrial dysfunction caused by HCV infection. Black-outlined boxes summarize the experimental evidence shown in this work with the downward distribution of mitochondrial dysfunctions suggesting a temporary progression of events. In blue possible connection with the HCV-induced ER-stress via Ca^{2+} ER-efflux/mitochondrial overload is shown. See text for further explanation.

Since a hallmark of defective activity in mitochondrial complex I is an enhanced electron leak, leading to superoxide formation, we measured, next, the intracellular reactive oxygen species production by specific probes. Fig. 5A shows that when assayed with MitoSox, a fluorescent probe specific for $O_2^{\bullet-}$ and that selectively accumulates in the mitochondrial compartment, the HCV-induced U-2 OS displayed a much higher signal than the non-induced cells. The overproduction of superoxide in the induced cells was also verified by the lucigenin–luminescence assay resulting in a 3 fold increase of superoxide production with respect to the basal level observed in the control cell (not shown). Analysis carried out with the probe, DCF, more specifically sensitive to H_2O_2, showed that in the induced cell, an even more evident production of H_2O_2 occurred and that it nicely localized to an intracellular compartment that resembled the mitochondrial network. Interestingly the production of H_2O_2 was delayed with respect to the establishment of the stationary level of the HCV proteins expression that was already reached after only 6–12 h of induction (data not shown). This would suggest an indirect effect of HCV protein on the observed oxidative stress caused by preceding alterations.

5. Aetiopathogenesis of HCV infection: a working hypothesis

A survey of the recent literature indicates that in cells transiently transfected with subgenomic constructs, some of the HCV proteins partially co-localize with mitochondria or are associated with a sub-fractions of mitochondrial preparations containing markers of the endoplasmic reticulum [10,12,13,18–24]. It has been shown by high resolution 3D reconstruction of confocal microscopy imaging that endoplasmic reticulum and mitochondria form an intertwined network with specific regions that are in tight contiguity [25,26]. These contact sites have been proposed to provide highly specialised areas enabling a functional/physical interaction between the two membranous systems. In particular, a localized calcium efflux from the ER into the intermembrane space might be taken up by mitochondria and used as signalling system [27,28]. In principle conditions that alter this calcium connection between ER and mitochondria can result in the deregulation of the mitochondrial physiology [29–32].

Appraisal of the data available combined with the evidence here reported tempts the proposal of the testable working model schematically shown in Fig. 6. Accumulation of HCV proteins in the ER membranes causes a local depletion of calcium (possibly by opening the ER Ca^{++}-channels) that is electrogenically accumulated by mitochondria causing depolarization of the membrane potential [33]. Although the increase of intramitochondrial calcium is known to activate a number of dehydrogenases that provides a prompt adaptative response to the stressing energy-requiring condition [34,35], this reaction that is beneficial if time controlled might instead results in deleterious effect when the insult is chronically maintained as in the case of the virus infection. High level of calcium can detach cytochrome c from the cardiolipin inner membrane [36] and activate the mitochondrial nitric oxide synthase with production of NO [37,38] that is known to be an inhibitor of complex IV [39,40] as well as of complex I [41] although by a different mechanism. The combination of these possible effects would result in an over-load of harmful reducing equivalents throughout the respiratory chain complexes and in an extra-production of ROS with respect to the basal level [29,30]. Once exhausted the buffering antioxidant capacity of glutathione and other redox buffers a self-fuelling cycle can be activated with further enhancement of reactive oxygen (nitrogen) species and alteration of the mitochondrial homeostasis [42,43]. These events, depending on the conditions, could, either culminate in the actuation of the apoptotic program [29,44,45], thus favouring diffusion of the virus infection or, paradoxically, result in a carcinogenic prosurvival reaction of the host-cell [46–49]. Pharmacological treatment of the infected cells with compounds able to restore the mitochondrial calcium homeostasis might prevent or even reverse the effects caused by HCV [19].

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