Activity increase of respiratory chain complexes by rubella virus with marginal indi- cation of oxidative stress

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Running title: Rubella virus alters activity of the respiratory chain

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Abstract

Mitochondria are important for the viral life cycle, mainly by providing the energy required for viral replication and assembly. A highly complex interaction with mitochondria is exerted by rubella virus (RV), which includes an increase in the mitochondrial membrane potential as a general marker for mitochondrial activity. This study aims at providing a more comprehensive picture of the activity of mitochondrial respiratory chain complexes I to IV. Their activity was compared among three different cell lines. A strong and significant increase in the activity of mitochondrial respiratory enzyme succinate: ubiquinone oxidoreductase (Complex II) and a moderate increase of ubiquinol: cytochrome c oxidoreductase (Complex III) was detected in all cell lines. In contrast the activity of mitochondrial respiratory enzyme cytochrome c oxidase (Complex IV) was significantly decreased. The effect on mitochondrial functions appears to be RV-specific as they were absent in control infections with measles virus. Additionally, these alterations of the respiratory chain activity were not associated with an elevated transcription of oxidative stress proteins and reactive oxygen species (ROS) were induced only marginally. Moreover, protein and/or mRNA levels of markers for mitochondrial biogenesis and structure were elevated such as nuclear respiratory factors (NRFs) and mitofusin 2 (Mfn2). Together, these results establish a novel view on the regulation of mitochondrial functions by viruses.
Introduction

Mitochondria are required for the maintenance of the cell function and integrity. Their most important role lies in energy production, but they are also at the intersection of regulatory pathways that coordinate metabolic processes (e.g. calcium homeostasis, cellular proliferation), cellular fate (apoptosis and necrosis) and antiviral defence (34), (31). Even a participation of mitochondria in the innate immune response was identified (34). There are a number of viruses that interfere with the important role of mitochondria in cellular antiviral response pathways, mainly with the regulation of apoptosis (31). Additionally, as the powerhouses of the cell, mitochondria provide most of the energy for viral replication and assembly. Up to 90% of the cellular ATP is produced in the inner mitochondrial membrane (IMM) by oxidative phosphorylation (OXPHOS), (40). OXPHOS comprises a series of redox reactions carried out by four multisubunit enzyme complexes (complex I to IV) of the electron transport chain (ETC). Electrons are transferred in a stepwise manner through this series of electron carriers from NADH (and FADH2) as reducing equivalents to the final acceptor molecular oxygen. A small percentage of electrons that are transported through the respiratory complexes leaks out, which results in generation of reactive oxygen species (ROS). The main ROS species is hydrogen peroxide, which is converted to water by enzymes such as catalase, peroxiredoxin or glutathione peroxidase as components of the cellular antioxidant system. Respiratory complex I and III are the main generators of mitochondrial ROS (8). The energy that is released during the flow of electrons is stored as an electrochemical proton gradient across the IMM, which is finally used by the ATP synthase (complex V) to generate ATP (40). A voltage potential, the mitochondrial membrane potential (Δψm), and a pH gradient are part of this proton motive force. Therefore Δψm serves as a general indicator for mitochondrial activity.

Mitochondria also participate in the assembly of membrane-associated viral replication complexes or might even function as the replication organelle itself. They also provide host replication factors (13). A prominent example for these replication factors is the mitochondrial matrix protein p32 (gc1q-R), (13), (21). Among its viral interaction partners is rubella virus (RV), an efficient teratogen and the only member of the Rubivirus genus within the family Togaviridae. The molecular mechanisms involved in the teratogenic outcome of RV infection of seronegative woman especially during the first trimester of pregnancy are not yet clear. RV itself exerts a
complex interaction with mitochondria. The RV capsid protein localizes to mitochondria and associates with p32 (2), which is required for RV replication (1). Additionally, the perinuclear accumulation of mitochondria in the proximity of RV replication complexes requires p32 (4). Moreover, the RV capsid protein is so far the only known RNA virus-encoded protein that interferes with protein import into mitochondria (17). While many viral infections affect the mitochondrial energy and oxidative balance, RV appears to exert an overall positive influence on mitochondrial and bioenergetic function. This is reflected by an increase in respiratory chain (RC) complex III activity, in the $\Delta \psi_{m}$, and by a high level of intracellular ATP in RV-infected cells in comparison to the mock-infected control even at late stages of infection (4).

In this study the modulation of mitochondrial respiratory chain complexes by RV was addressed systematically. The results shown indicate that in the course of RV infection the activity of all four RC complexes is significantly altered in three different cell lines irrespective of their metabolic (tumorogenic and non-tumorogenic) background. Especially the increase in respiratory complex II activity was profound revealing an increase by up to 141% compared to the control. These alterations appear to be RV-specific as they were absent after application of measles virus (MV) as control virus. Additionally, the increase of complex II activity was only marginally associated with oxidative stress induction. A mechanism for the positive influence of RV on the ETC is discussed. The results presented reveal a complex interaction of RV with mitochondria, which ensures ongoing supply of energy during its long replication cycle. A deeper understanding of the modulation of mitochondrial respiratory complexes by viruses such as RV could contribute to our understanding of mitochondrial functions and the anti viral treatment of these viruses with metabolic antagonists (11).
Materials and methods

Reagents
Digitonin was purchased from Fluka Biochemica (Ulma, Germany). All other reagents and general lab chemicals were from Sigma Aldrich (Hannover, Germany). Media, supplements for cell culture, and fetal calf serum (FCS) were purchased from Life Technologies-Invitrogen (Darmstadt, Germany). A549 and Vero cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were obtained from IAZ (Munich, Germany).

Cell culture and virus infection
The epithelial cell line MCF-7 was maintained in Eagle’s minimum essential medium (MEM) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 10% FCS and antibiotics. Vero cells and A549 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) with antibiotics. Medium for A549 cells was supplemented with 1 mM HEPES. All cell lines were cultivated at 37°C under 5% CO₂ in a humidified incubator. All cell lines were either mock-infected (control) with medium excluding virus or infected with RV wild-type strain Therien at a multiplicity of infection (MOI) of 5 plaque forming units (PFU) per cell. MV laboratory strain Edmonston was applied to A549 and MCF-7 cells at a MOI of 5 and to Vero cells at a MOI of 0.01. Fresh medium was added after 2 hours of incubation at 37°C. Assays were performed at 12, 24, 48 and 72 hours post-infection (hpi). Viral titers were determined by standard plaque assay as described (5).

Isolation of a mitochondrion-enriched cellular fraction
To obtain a mitochondria-enriched cellular fraction, intact mitochondria were isolated from 60 mm dishes in a medium of 0.2 mM EDTA, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.8 (mito buffer) by differential centrifugation of cell homogenates obtained after mechanical disruption of cells (MagNA Lyser, Roche, Mannheim, Germany). Differential centrifugation was done according to published protocols (MitoSciences, www.Mitosciences.com/PDF/mitos.pdf and Sigma Aldrich, technical bulletin for mitochondria isolation kit). Briefly, 10 mM triethanolamin and 0.1 mg/ml digitonin were added before homogenization. Undisrupted cells and nuclei were removed by centrifugation at 1,000 × g for 10 min at 4°C. Mitochondria were pelleted from the supernatant by centrifugation at 3,500 × g for 15 min at 4°C and subsequently solubilized in 100 µl mito buffer supplemented with 0.5 mM PMSF and 0.05 mM...
pepstatin A. Protein concentration was determined by the bicinocinonic acid test (BCA). The yield was typically 80 to 150 µg (in a total volume of 100 µl) per 60 mm dish of cultured cells.

Assays for the activity of respiratory complexes I to IV

Freshly prepared mitochondria (10 µl of a 0.4 µg/µl mitochondrial fraction for CI, III, and IV, and 10 µl of an undiluted mitochondrial preparation for CII) were used for the spectrophotometrical determination of the activity of RC complexes I to IV by biochemical assays with a total volume of 200 µl. Protocols were adapted from previous publications [(27) and (19) for complex I and II; (24) for complex III; and (27) for complex IV]. Activities of respiratory chain complexes were calculated as units per 1 µg of isolated mitochondrial fraction and then normalized to citrate synthase activity. **Complex I, NADH: ubiquinone oxidoreductase activity** was determined in assay-buffer composed of 25 mM potassium phosphate, 3.5 mg/ml BSA, 0.06 mM DCPIP, 0.0125 mM antimycin A, and 0.7 mM decylubiquinone. Reaction was started by adding 0.2 mM NADH. CI activity was measured spectrophotometrically by the oxidation of NADH with decylubiquinone at 600 nm. **Complex II, succinate: ubiquinone oxidoreductase activity** was determined in assay buffer containing 25 mM potassium phosphate, 2 mg/ml BSA, 20 mM succinate, 0.05 mM DCPIP, 20 mM NaN₃, and 0.1 mM ATP. Reaction was started through the addition of 0.1 mM decylubiquionone. Complex II activity was assessed spectrophotometrically by the oxidation of succinate with ubiquinone at 600 nm. **Complex III, ubiquinol: ferrocytochrome c oxidoreductase activity** was assessed in assay buffer containing 50 mM Tris HCl, 4 mM NaN₃, 0.1 mg/ml BSA, 0.05% (v/v) Tween-20. Reaction was started through addition of 0.25 mM decylobiquinol and 0.0625 mM cytochrome c. Reduction of cytochrome c with decylubiquinol was determined at 550 nm. **Complex IV, cytochrome c oxidase activity** was determined through addition of 150 µl of reduced cytochrome c (50 µM) to 10 µl of diluted mitochondrial fraction. Complex IV activity was measured by the oxidation of reduced cytochrome c at 550 nm.

Measurement of ROS

Intracellular ROS generation was detected using the probe 2', 7'-dichlorofluorescein diacetate (DCF-DA) as a nonfluorescent cell-permeable compound. Intracellular esterases cleave the acetate groups, such that the nonfluorescent dye 2',7'-
dichlorofluorescein (DCF) is retained intracellularly, which in turn is oxidized by intracellular ROS (mainly by hydrogen peroxides) and thus becomes fluorescent. The fluorescent product DCF was detected with an excitation of 485 nm and an emission of 530 nm on a fluorescence microscope (DM IRB, Leica, Wetzlar, Germany) and a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. Vero cells were plated at a density of $1 \times 10^6$ per 60 mm dish and after 24 hours mock- and RV-infected. At 72 hours post-infection (hpi) Vero cells were incubated with DMEM containing 1% FCS and 70 µM DCF-DA for 30 min at 37°C in the dark. After washing with PBS cells were trypsinized, pelleted and resuspended in DMEM with 1% FCS. After an incubation period of 60 min under shaking conditions, cells were pelleted and fixed with 2% (w/v) PFA and resuspended in PBS. Thereafter intracellular ROS generation was measured by flow cytometry. Three independent samples of 10,000 cells were analyzed for each experimental condition, and the percentage of RV-infected cells with high DCF fluorescence was calculated in relation to the corresponding mock sample as described. As negative controls both mock-infected, unstained and mock-infected, dye-loaded Vero cells were employed. As a positive control, oxidative activity was increased through preincubation of mock-infected Vero cells with 30 µM oligomycin and 0.03% H$_2$O$_2$ for 15 hours before loading with the DCF-DA dye. For fluorescence microscopy analysis, Vero cells were loaded with DCF-DA as described above. During an additional incubation period of 1 hour at 37°C cells were incubated with 5 µg/ml Hoechst bisbenzamide 33285 (Invitrogen). Thereafter cells were directly subjected to microscopic analysis. Postacquisition analysis was performed with Corel Photo-Paint 11 software with minimal alterations to background and contrast, which were applied equally to samples and controls.

**Western blot analysis**

Equal amounts (30 to 70 µg) of mitochondria-enriched fractions were separated on a 10% bis-acrylamide gel electrophoresis gel under denaturing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking of the membrane with 5% dry milk, probing was performed with a 1:200 dilution of the rabbit polyclonal antibody against Mfn2 and the rabbit polyclonal antibody against VDAC3 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), a 1:200 dilution of the polyclonal antibody to VDAC and a 1:500 dilution of the rabbit SDHA polyclonal
antibody (Acris antibodies, Herford, Germany) overnight at 4°C. Incubation with secondary antibody and detection by chemiluminescence were as described (4).

**Gene expression analysis by quantitative real-time PCR**

RNA was prepared and samples were processed as described previously (3). Briefly, total RNA was isolated by TriFast™ reagent (PeqLab, Erlangen, Germany) and 1.25 µg of RNA were reverse transcribed. Quantitative real-time PCR (RT-qPCR) was performed with SYBR green dye technique on a Light Cycler system (Roche) to determine the mRNA expression level of heme oxygenase-1 (HO-1), nuclear factor of the kappa light chain enhancer of B cells (NF-κB), nuclear encoded subunit NDUFB1 of complex I, subunit A and B (SDHA and SDHB) of complex II [succinate dehydrogenase (SDH)], subunit UQCRC1 of complex III, and COX4I1 of complex IV, and nuclear factor E2-related factor (NRF) 1 and 2. Gene expression was calculated with geNorm version 3.5 and normalized to the housekeeping genes hypoxanthine phosphoribosyltransferase 1 (HPRT1) and HUEL (solute carrier family 30 member 9).

For quantification of viral subgenomic and genomic RNA 1 µg of RNA was reverse transcribed and the primer pair TBR2 and TBR7 was employed to amplify a 177 bp region from the capsid protein. A plasmid standard curve was generated in the range of 1×10⁹ to 1×10² based on the RV infectious cDNA clone Robo503 (a kind gift of Dr. Frey, Georgia State University, Atlanta, USA). The standard curve was used to determine the copy number of viral RNA in each respective sample. Primer sequences are given in table 1. Primer specificity for the selected cellular genes was confirmed by sequence analysis.

**Statistical analyses**

All data in the diagrams are expressed as means ± SE of at least three individual experiments. Level of significance from control population is indicated by an asterix (* < 0.05, ** < 0.01, and *** < 0.001). Standard two-tailed paired student's t-tests were used for evaluating the significance of the difference between the means of RV- and mock-infected cultures.
Results

Effects of RV infection on the activity of respiratory chain complexes I to IV

Three RV-permissive cell lines with a different metabolic background (Vero as a non-tumorogenic cell line, the human breast adenocarcinoma cell line MCF-7 and the human lung carcinoma cell line A549) were used for a comprehensive analysis of the activity of RC complexes under RV-infection. MOI 5 was chosen to achieve a high initial infection rate. All three cell lines are susceptible to high replication of RV, however, the replication kinetics differs among these three cell lines (figure 1A). A549 cells are characterized by a high initial RNA load already observable at 12 hours post-infection (hpi). In contrast to A549 cells, in Vero and MCF-7 cells viral RNA reached a plateau and was maintained at a high level until 72 hpi. All three cell lines were mock-infected and the activity of RC complex I to IV was determined after an incubation period of 24, 48, and 72 hours (hrs). Figure 1B indicates that the activity of RC complex I exceeds the activity of the remaining complexes. MCF-7 cells possess a slightly higher activity of complex II. However, the activity rate of all four RC complexes is similar between the three cell lines tested. Thereafter Vero, MCF-7, and A549 cells were infected with RV and samples were collected at 12, 24, 48 and 72 hours post-infection (hpi) in order to determine RC complex activity as infection proceeds (corresponding mock-infected controls were set to 100%). Figure 2 and table 2 show the alterations in the activities of mitochondrial respiratory chain (RC) complexes, which is further summarized in table 2. Peak activities of respiratory chain complexes I to IV and the time point of RV-induced alterations were determined for the indicated cell lines. RV-induced alterations in the activities of complexes I to IV were similar between the three cell lines tested, especially between Vero (figure 2A) and MCF-7 (figure 2B) cells. However, Vero (decrease to 64% ± 10%) and MCF-7 (increase to 145 ± 14%) cells differ in the activity of RC complex IV at 48 hpi. In all three cell lines tested, alterations were only noticeable after 12 hpi. Complex I and IV are apparently least altered in RV-infected cells. However, complex I activity is strongly reduced in RV-infected A549 cells to 53% ± 5% at 72 hpi (figure 2C). In all three cell lines tested, complex II represents the main target of RV-induced alterations within the ETC. In comparison to the mock-infected population, its activity was significantly increased by RV infection by 141% (to 241% ± 15%, A549), by 131% (to 231% ± 31%, MCF-7), and by 99 (to 199% ± 31%, Vero). Hence the extent of up-regulation of complex II activity was very similar between the three cell lines.
The only noticeable difference in complex II activity between RV-infected Vero and MCF-7 (72 hpi) cell versus A549 cells (24 hpi) lies in the time point of complex II peak activity. Complex IV activity except at 48 hpi for MCF-7 cells is significantly down-regulated in all three cell lines. The highest level of down-regulation of complex IV by 46% (to 54% ± 15%) was observed for A549 cells. The activity of complex III was moderately increased by 60% (to 160% ± 27%, A549), by 42% (to 142% ± 23%, MCF-7), and by 44% (to 144% ± 17%, Vero). The same applies for complex I with a slight increase between 22% (increase to 122% ± 26%, Vero) and 53% (increase to 153% ± 26%, A549). MCF-7 cells, however, showed no increase in complex I activity.

The next attempt was to determine the specificity of these alterations. As a control virus, MV strain Edmonston was used, as all three cell lines applied in this study are susceptible to MV. MV like RV replicates in the cytoplasm, but in contrast to RV is not known to be associated with mitochondria. Comparable to RV, MCF-7 and A549 cells could be infected with MV at a MOI of 5. Due to extensive syncytium formation Vero cells require a lower MOI of 0.01. Samples were collected at 24 and 48 hpi, as MV induces a strong cytopathic effect after 48 hours of infection. Just with a few exceptions the activity of most RC complexes remains almost unchanged after MV-infection (figure 3). Among the three cell lines tested MCF-7 showed the strongest alterations, which is reflected by an increase in complex I, II, and IV activity at 48 hpi by 32% (to 132% ± 1%), by 34% (to 134% ± 15%), and by 34% (to 134% ± 12%), respectively. In Vero cells only complex IV activity was increased by 69% (to 169 ± 15% at 48 hpi. The increase in complex IV activity in Vero cells could be due to extensive syncytium formation, which was not observable in A549 and MCF-7 cells. Syncytium formation was reduced after application of MV at a MOI of 0.001, which also lacked the induction of complex IV activity (data not shown).

In summary, the RV-induced alteration of the activities of RC enzymes is virus-specific, complex and occurred in three different cell lines in a similar manner and to a similar extent. Especially for complex II a strong increase to up to 231% (± 31%) in its activity was observed.

Gene expression analysis of oxidative stress markers

RV infection results in a higher activity of respiratory complexes, especially of complex II (figure 2). Based on these results we hypothesized that the high mitochondrial activity in RV-infected cells could be accompanied by oxidative stress induction. Hence the effect of RV infection on the mRNA expression of two
representative markers for oxidative stress induction was measured by RT-qPCR in Vero and A549 cell lines as they are the most frequently used cell lines for RV infection studies. The nuclear factor of the kappa light chain enhancer of B cells (NF-κB) regulates as a transcription factor various cellular processes, including immune and stress response and proliferation (33). The predominant form within the mammalian NF-κB family is a heterodimer of family member p65 and p50. Heme oxygenase is a cellular stress protein required for the cleavage of heme to generate biliverdin, a potent antioxidant. Heme oxygenase-1 (HMOX-1) represents its inducible isoyme, which is activated by various stimuli including oxidative stress, heat shock, and inflammatory agents (22). Figure 4 shows no significant increase in the mRNA expression of NF-κB/p65 in Vero cells and of HMOX-1 in Vero and A549 cells. Only A549 cells displayed an alteration in the mRNA expression of NF-κB/p65 in comparison to the mock-infected controls (set to 100%). The increase in the NF-κB/p65 mRNA level was already significant on the first day of infection (to 212 % ± 27%) and increased further until the third day of infection (to 304% ± 111%). To confirm the suitability of NF-κB and HMOX-1 as oxidative stress markers, the respiratory complex V (ATP synthase) inhibitor oligomycin (30 µM) as an inducer of oxidative stress was applied to mock-infected Vero and A549 cells at 48 hpi. Oligomycin-treatment resulted in an upregulation of the mRNA expression of NF-κB and HMOX-1 with the exception of HMOX-1 in A549 cells (figure 4). However an increase in HMOX-1 mRNA expression was detected after treatment of A549 cells with 0.03% hydrogen peroxide (data not shown). Hydrogen peroxide was not included as an inducer of oxidative stress in figure 4, as there was a broad range of variation in HMOX-1 and NF-κB mRNA expression. These data indicate that RV-infected cells lack an upregulation of oxidative stress markers. As a next step, this data was to be complemented by the determination of reactive oxygen species (ROS) generation as a second and important parameter for oxidative stress induction.

Evaluation of ROS generation
An enhanced electron flow rate could lead to increased electron leakage and finally oxidative stress through generation of ROS. Mainly respiratory complex I and III contribute to generation of ROS (8). To determine therefore whether RV-infection is accompanied by production of ROS mock- and RV-infected Vero and A549 cells were incubated at 72 hpi with the dye DCF-DA. This time frame was selected...
because of the time point of the highest increase of complex I, II and III activity in RV-infected Vero cells, which lacked an increase in mRNA expression level of selected markers for oxidative stress induction (figure 4). Thus if ROS is generated, it should be detectable at this time point. Additionally, at 72 hpi a cytopathic effect (indicative for cytopathogenicity) is clearly visible in RV-infected Vero and A549 cells (data not shown).

The ROS-dependent generation of the fluorescent compound DCF was measured by both flow cytometric (figure 5A and C) and microscopic analysis (figure 5B and D). As a positive control, Vero and A549 cells were pretreated with the oxidative stress inducers oligomycin (30 µM) or 0.03% H₂O₂ (0.03%) for 15 hours. In comparison to the untreated control, both aforementioned inducers of ROS resulted in a strong increase in intracellular DCF fluorescence (figure 5). In comparison to the mock-infected controls, the flow cytometric analysis revealed for RV-infected Vero and A549 cells (72 hpi) only a minimal increase in ROS levels (figure 5A and C, respectively). The extent of ROS generation was comparable between Vero and A549 cells. There was only a slight shift in DCF fluorescence detectable. To be able to calculate the shift in DCF-fluorescence in flow cytometry histograms and to compare the results of three independent experiments, the marker M1 was set such that in the right portion of the histogram 50% of the total DCF-DA stained and untreated Vero and A549 (mock) cell population was encompassed. The marker was copied to histograms of oligomycin (30 µM) or 0.03% H₂O₂ pretreated or RV-infected Vero and A549 cells cells. Figure 5A and 5C also include the mean (± SE) for the marker M1 obtained for the indicated samples in three independent experiments. For Vero cells, the M1 marker included 60% (± 2%) of the total cell population for an infection with RV (MOI=5). For A549 cells, the M1 marker included 66% (± 7%) of the total cell population for an infection with RV (MOI=5). This indicates that RV-infection results only in a slight increase of DCF fluorescence. Pretreatment with 30 µM oligomycin or 0.03% H₂O₂ resulted in Vero cells in an increase in the 50% population included by the marker M1 by 36% (to 86% ± 10%) or by 24% (to 74% ± 6%), and in A549 cells in an increase by 39% (to 89% ± 7%) or by 26% (to 76% ± 6%), (figure 5A and C, respectively). The generation of ROS was also analyzed by fluorescence microscopy. Figure 5B and 5D illustrate the slight increase in ROS generation in RV-infected Vero (figure 5B[b]) and A549 (figure 5D[b]) cells in comparison to the mock-infected population (figure 5B[a]) and (figure 5D[a]), respectively. In contrast to RV,
pretreatment of Vero and A549 cells with 30 µM oligomycin resulted in a strong increase in intracellular ROS levels (figure 5B[c]) and (figure 5D[c]). Hence, despite a significant increase in the activity of selected mitochondrial respiratory complexes RV infection induces only a moderate increase in intracellular ROS as compared to the mock-infected cell population.

**Analysis of the expression of transcription factors regulating mitochondrial respiratory function and of subunits of respiratory complex II**

In an attempt to identify factors that could contribute to the observed changes in mitochondrial activity, the mRNA expression of master regulators of mitochondrial biogenesis and function and of subunits of RC complexes was determined. As representative master regulators, the nuclear respiratory factor (NRF) 1 and 2 were chosen. They are key nuclear transcription factors, which regulate the expression of nuclear genes required for mitochondrial respiratory function and mitochondrial DNA transcription and replication. Their gene targets also include subunits of the five respiratory complexes. While RC complexes I, III, IV are encoded by mitochondrial and nuclear DNA, complex II is entirely nuclear encoded (42). The effect of RV infection on the mRNA expression of selected nuclear encoded subunits of the RC complexes I, II, III and IV was examined. For this purpose the NDUFB1 subunit of complex I, the subunit A and B (SDHA and SDHB) of complex II [succinate dehydrogenase (SDH)], the subunit UQCRC1 of complex III, and the COX4I1 subunit of complex IV was selected. The mRNA expression profile of these genes was investigated in A549 and Vero cells at 24, 48, and 78 hours post-infection. RV-infected samples were compared to the corresponding mock-infected control (set to 100%), (figure 6A and B). The mRNA expression profile of NRF1 and 2 showed a similar increase in both cell lines (figure 6A). The NRF2 gene was the most significantly up-regulated gene. In comparison to the mock-infected control, NRF2 mRNA expression increased by 115% (to 215% ± 16%, A549) and by 151% (to 251% ± 21%, Vero) over time of infection. The mRNA of the NDUFB1 subunit was downregulated at all selected time points by up to 43% (to 57% ± 2%) in Vero cells at 48 hpi and by up to 29% (to 71% ± 7%) in A549 cells at 48 hpi (figure 6B). At 24 hpi a significant increase over the mock- infected population was detected for the mRNA expression of the SDHA subunit of complex II in both, Vero (to 165% ± 19%) and A549 (to 200% ± 4%) cells. However, at 72 hpi a significant decrease by 39% (to 61% ± 17%) was detected for SDHA in Vero cells. A significant increase (to 169% ±
14% [A549 at 24 hpi] and to 161 ± 14% [Vero at 72 hpi]) was seen in both cell lines for the expression of the SDHB gene (Figure 6B). Hence only the time point of the highest mRNA expression level of SDHB varied between both cell lines. The mRNA level of the UQCRC1 subunit of complex III was only increased in A549 cells at 24 hpi by 46% (to 146% ± 9%). The mRNA expression level of the COX4I1 subunit of complex IV was increased at 24 hpi in both cell lines, Vero (to 137% ± 21%) and A549 (to 129% ± 23%). The data presented shows, that RV-induces alterations of the mRNA expression of NRF1 and 2 as key regulators of mitochondrial biogenesis and of selected RC subunits as their subsequent targets. Among the examined RC subunits the mRNA expression changes were most profound for the SDHA and SDHB subunits of complex II. Hence the next attempt was to determine the protein expression level of SDHA in mitochondria-enriched fractions of Vero (figure 6C) and A549 (figure 6D) cells by western blot analysis. In A549 cells no changes in the protein expression of SDHA were detected after RV-infection in comparison to the mock-infected control. However, the picture was different for Vero cells. At 48 and 72 hours post-infection an additional band of higher molecular weight was seen only in RV-infected samples (figure 6C). In summary, RV-infection alters the mRNA expression level of SDHA in Vero cells and of selected subunits of RC complexes, most notably of complex II. This is also reflected by an altered protein expression state, possibly corresponding to heterodimer formation.

Expression of mitofusin 2 as a marker for mitochondrial morphology and metabolism

The peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α belongs to a family of transcription coactivators and as such regulates several key metabolic processes. PGC-1α induces the transcription of the aforementioned transcription factors NRF-1 and NRF-2 (42), but also of mitofusin 2 (Mfn2) as an important regulatory protein for mitochondrial structure and metabolic activity (52). Therefore the next attempt was to determine the mRNA and protein expression level of Mfn2 in RV-infected Vero and A549 cells. Figure 7A shows that the mRNA expression of Mfn2 was significantly elevated at 72 hpi in A549 (to 773% ± 168%) and Vero (to 151% ± 51%) cell lines. As Mfn2 is a mitochondrial protein, a mitochondria-enriched cellular fraction was used for analysis of its protein expression level. Figure 7B and C suggest differences in the mitochondrial Mfn2 protein expression level over time of infection. Western blot analysis of isolated mitochondria shows that at 3 days post-
infection mitochondria in RV-infected Vero and A549 cells have a higher portion of Mfn2 as compared to the mock-infected control. Additionally, the effect of heat stress (as an inducer of oxidative stress) on Mfn2 expression was examined in Vero cells. Therefore Vero cells were kept under 42°C for 3 hours and then incubated for 24 hours at 37°C and subjected to isolation of a mitochondria-enriched cellular fraction. Western blot analysis of this fraction from HS-induced Vero cells revealed a higher expression level of Mfn2 in comparison to the control population (figure 7C). These results indicate that RV infection induces an upregulation of Mfn2 at its mRNA and protein level in two representative, RV-permissive cell lines.
Discussion

As intracellular parasites viruses rely on their cellular host for providing the frame, the building blocks and the energy required for its replication machinery. Hence viruses pose a burden on the infected cell, especially through exhaustion of its metabolism. Data presented here indicate, that RV as a slow-replicating virus has evolved means to ensure ongoing energy supply for its replication while circumventing the induction of oxidative stress. This clearly distinguishes RV from other viruses. Evidence is provided in this paper that RV-infection strongly upregulates the activity of RC complex II and leads to an increase of protein and/or mRNA expression level of markers for mitochondrial biogenesis and function. This is in line with the presumption in our previous publication where it was shown that the influence of RV on cellular metabolism is rather positive. In comparison to mock-infected controls the mitochondrial membrane potential and the intracellular ATP content in RV-infected cells was increased (4).

Despite a renewed focus on metabolic engineering by viruses (26), data on the underlying mechanism remain to be gained. Some viruses such as influenza and measles virus do not rely on cellular metabolism (37) or mitochondrial respiration (6), respectively. However, in most cases, virus infection leads to mitochondrial dysfunction and subsequently to induction of oxidative stress. This has been shown for rabies virus-infected mouse dorsal root ganglion neurons, where induction of oxidative stress contributes to the reduced axonal outgrowth (18). Moreover, in Dengue virus-infected hepatocytes $\Delta \psi_m$ and intracellular ATP content are decreased indicating mitochondrial dysfunction (12). Also in Hepatitis C virus (HCV) infected cells massive generation of ROS and a decreased $\Delta \psi_m$ were described (34). Mitochondrial dysfunction and ROS generation were observed 24 hours after infection of a mouse neuroblastoma cell line by Sindbis virus, the prototype member of the alphavirus genus within the family Togaviridae (44). In contrast to these observations we found that the metabolic activities of mitochondria are maintained through the entire RV replication cycle. Even at the clearly visible onset of the cytopathic effect at 72 hpi, the activity of distinct RC complexes is up-regulated.

Our investigations show comparable results on the activity of the respiratory chain in three different RV-infected cell lines highlighting complex II as the main target of the
metabolic modulation by RV. This is especially noteworthy as breast cancer cells such as the MCF-7 cell line are characterized by higher OXPHOS rates and a higher activity of RC complex II (48). There are only a few investigations on the modulation of the activity of RC complexes by chemical or natural compounds or by viral infections. One such example is melatonin that has antioxidant functions and increases the activity of RC complexes I and IV probably through enhancement of the electron flow along the ETC (32). Streptozotocin-induced diabetic rats are characterized by an increase in the activity of respiratory complexes I and II and a decrease in complex III and IV activities (35). HCV-infected cells show an impairment of OXPHOS, which appears to be mainly due to a reduction in the activity of RC complex I by up to 50% (36). The respiratory capacity of complex I and II decreases when Sindbis virus infection of a mouse neuroblastoma cell line proceeds (44). An increase in the activity of both, the respiratory complex IV and the ATP synthase, has been described after HIV-1 infection (46). Mouse models for coxsackievirus B3-induced myocarditis suggest an involvement of mitochondria in virus elimination and viral pathology (10). In this regard the elevated activity of RC complex I (by 21.7% ± 6.1%) and III (by 44% ± 10.3 %) appears to contribute to generation of ROS and subsequently to mitochondrion-related apoptosis and virus elimination (10). However, the observed alterations of respiratory chain complex activities in response to RV infection appear to be much more profound and more interestingly, show only marginal induction of markers for oxidative stress such as ROS. Respiratory complex I and III are the main generators of mitochondrial ROS (8). Hence the up-regulation of RC complex II by RV should be favourable in the context of ROS generation. The increase in the mRNA expression level of NF-κB (as a marker for oxidative stress) in RV-infected A549 cells is probably due to an antiviral interferon response. Vero cells lack the type I interferon response (7), which particularly activates the NF-κB pathway (33).

In the three cell lines tested a downregulation of complex IV activity was detected at some time point of infection with RV. There are two possibilities for a compensatory modulation of RC complex activities. First, the downregulation of complex IV could be an adaptive response to the increase in complex II activity to avoid an overfunctioning of the respiratory chain. Second, the downregulation of complex IV might be compensated by an increase in complex II activity to ensure ongoing electron flow through the electron transport chain. Similar compensatory mechanisms
were also reported for cell lines derived from patients with an ATP synthase deficiency (complex V) which was balanced by an up-regulation of RC complex III and IV activity (15). Similar to RV infection (but at a different scale), ionizing radiation of A549 cells induces an up-regulation of complex II activity (by about 23%) and a down-regulation of complex IV activity (by about 12%), (49).

There are several possibilities to explain how RV modulates the activity of the respiratory chain. RV infection results in slight alterations of the mRNA expression level of subunits of complex I, III, and IV, whereas the mRNA expression level of SDHA and SDHB shows the highest rate of induction among the RC subunits and especially SDHB is kept at an almost constantly increased level in Vero cells. This supports the important role played by respiratory complex II during the course of RV infection. Complex II is the only enzyme that participates in both, the tricarboxylic acid (TCA) cycle and the respiratory chain. This may subsequently lead to an enhancement of the TCA cycle, because all enzymes of the TCA cycle react as a group of enzymes and form a metabolon. The TCA cycle fuels the respiratory chain with reducing equivalents. Higher mRNA levels of the SDHA subunit of complex II in RV-infected cells seems to result in an altered SDHA protein expression state. A higher molecular weight species of SDHA appears on western blots (figure 6C) together with the increased complex II activity at 48 hpi. This higher molecular weight species is absent in mock-infected Vero cells and in RV- and mock-infected A549 cells. A549 cells show the strongest upregulation of complex II activity already at 24 hpi. Hence a higher molecular weight species of SDHA could possibly not have been formed. The mechanism of RV-induced upregulation of complex II activity in Vero cells appears to differ from the one found in A549 cells. The higher molecular weight species of SDHA might correspond to heterodimers formed with SDHB. SDHA and SDHA both represent the catalytic moiety of complex II and thus RV-infection could support heterodimer formation, which could result in higher complex II activity. In A549 cells the viral RNA level is at 12 hpi higher than the one found in Vero or MCF-7 cells, which could contribute to the observed early increase in respiratory complex II activity in A549 cells at 24 hpi. We could not find differences in the inherent activity of respiratory complexes I to IV in mock-infected A549, MCF-7, and Vero cells (figure 1A), that could help to explain the slight variations in respiratory complex activity between Vero and A549 cells after RV infection (figure 2). The activity rate of each complex of the RC is comparable between Vero, A549, and MCF-7 cells. Important
contributors to RV-induced alterations of RC activities appear to be the transcription factors NRF1 and NRF2. The mitochondrial respiratory machinery is regulated by both, nuclear and mitochondrial genes. Their coordinated action is ensured by PGC-1 family of coactivators, which in turn activate transcription factors such as NRF1 and NRF2 (41). NRF1 and NRF2 have overlapping, but also distinct functions. NRF1 mainly coordinates the expression of genes that are necessary for both, mitochondrial respiration (including genes encoding RC subunits) and the DNA replication apparatus (42). An elevation of NRF-1 mRNA is already sufficient to stimulate mitochondrial activity (42). Especially NRF2 is involved in the stress response pathway and regulates the expression of antioxidant enzymes (30). Both, RV-infected Vero and A549 cells show an elevated mRNA expression level for NRF-1 and NRF-2. Hence both transcription factors probably contribute to the positive effect of RV on mitochondrial activity, and especially NRF-2 to the low level of oxidative stress induction. How an altered activity rate of complex II could be beneficial for RV and could possibly contribute to the previously observed increase in mitochondrial membrane potential and the intracellular ATP content in RV-infected cells (4) remains to be resolved. The data presented here add to the complexity of known and unknown functions of complex II. The respiratory chain complexes are not a linear arrangement of enzymatic supercomplexes, they show different activity rates among each other (figure 1B) and also tissue-specific activity rates and expression levels (9). Each subunit of the RC complexes differs in its ability to relate its protein expression level to its enzymatic activity (38). For example one third of the regular protein content of the 8 kDa subunit of complex I is sufficient to maintain 70% of regular complex I activity. More importantly, complex IV activity has to decrease to a critical value until mitochondrial respiration in general is effected (38). This so called biochemical threshold effect is specific for each complex. During the initial stages of physiological stress an activation of SDH activity was noticed in rat blood lymphocytes (50). Hence an increased activity of complex II could be beneficial under physiological or metabolic stress, which is also posed on the infected cell by RV. Defects in complex II functions are relatively rare in humans and complex II is the only component of the RC that is capable of maintaining its activity under reduction of the RC and in the presence of ATP (39). Moreover, complex II can also induce
reverse electron flow through complex I, which in turn supports NADH production (39). This emphasizes the importance and unique properties of complex II.

Another important point to consider is how the reported interference of RV with mitochondrial protein import fits into this picture (17). This phenomenon was so far only demonstrated in yeast cells and certainly needs further investigation. The protein import and assembly process of mitochondrial subunits is complex and requires several cofactors, which to some extent differ among yeast and mammalian cells (28). Moreover, protein import into mitochondria requires the translocase of the outer membrane (TOM). This also includes the import of most of the subunits of the RC complexes (20). However, surface-exposed TOM receptors are not required for the interference of RV with mitochondrial protein import (17).

Besides the regulation of NRF1 and NRF2 transcriptions factors, PGC-1-dependent pathways positively influence mitochondrial activity and biogenesis and are thus involved in the adaptation of mitochondrial metabolism to the energy needs of the cell. Mfn2 represents one of the targets of the nuclear coactivator PGC-1α (52). Mfn2 is a dynamin-related GTPase protein that fulfills several important functions within the cell in addition to direct mitochondria fusion. Mfn2 expression is increased at mRNA and protein expression level after RV infection (figure 7). The slight discrepancy between the constant increases of Mfn2 at its mRNA level in A549 cells to its decreased protein expression rate at 48 hpi might not be significant. This discrepancy could be due to differences in Mfn2 expression rate between Vero and A549 cells or an impaired import rate of Mfn2 into mitochondria at 48 hpi. Expression of Mfn2 is induced in skeletal muscle by acute exercise (52) and by mild heat stress (23). Mfn2 was also reported to protect against cold stress, which is associated with induction of apoptosis and reactive oxygen (51). The Vpr protein of human immunodeficiency virus (HIV-1) reduces the expression level of Mfn2, which results in loss of mitochondrial membrane potential and apoptotic cell death (16). Moreover, Mfn2 gain-of function increases $\Delta \psi_m$ and stimulates mitochondrial activity (52), which was also seen in RV-infected Vero and MCF-7 cells (4).

The low level of oxidative stress induction in RV-infected cells is consistent with the recent observation that in response to stress including oxidative stress and virus infection cells generate Ras-GAP SH3 domain-binding protein (G3BP)-containing stress granules. However, these granules were only detectable in less than half of the infected cells and only at late stages of RV-infection (25).
Taken together, the data presented here indicate that RV has evolved a mechanism through which mitochondrial functions are positively influenced. This occurs without the usually during a virus infection inevitable induction of oxidative stress. The study of viral modulation of mitochondrial functions could not only reveal important aspects on signalling and metabolic pathways that are involved in the regulation of mitochondrial activity. The alteration of mitochondrial respiratory and redox functions by viruses such as RV could contribute to therapeutic strategies comprising metabolic antagonists against viral infections. Even metabolic agonists for compensation of mitochondrial defects without oxidative stress induction could be designed (11), (47).

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References


45. **Sun, W., M. M. Guo, P. Han, J. Z. Lin, F. Y. Liang, G. M. Tan, H. B. Li, M. Zeng, and X. M. Huang.** 2012. Id-1 and the p65 subunit of NF-kappaB promote migration of nasopharyngeal carcinoma cells and are correlated with poor prognosis. Carcinogenesis 33:810-817.


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**Figure legends**

Figure 1. (A) Analysis of the replication kinetics of RV in Vero, MCF-7, and A549 cells and (B) the activities of mitochondrial respiratory chain complexes I, II, III,
Mean RV genomic and subgenomic RNA copies were determined for 12, 24, 48, and 72 hours post-infection (hpi) by RT-qPCR. Enzyme activities were determined by spectrophotometrical assays and normalized to citrate synthase activity. Vero, MCF-7, and A549 were mock-infected after 24 hours of cultivation. Samples were collected at further incubation periods of 24, 48, and 72 hours (hrs).

**Figure 2.** Activities of mitochondrial respiratory chain complexes I, II, III, and IV were measured in mitochondria isolated from RV-infected Vero (A), MCF-7 (B), and A549 (C) cells. Enzyme activities were determined by spectrophotometrical assays and normalized to citrate synthase activity. Data are shown as percentages of the corresponding mock-infected controls, which were set as 100%. hpi, hours post-infection

**Figure 3.** Activities of mitochondrial respiratory chain complexes I, II, III, and IV were measured in mitochondria isolated from MV-infected Vero (A), MCF-7 (B), and A549 (C) cells. Enzyme activities were determined by spectrophotometrical assays and normalized to citrate synthase activity. Data are shown as percentages of the corresponding mock-infected controls, which were set as 100%. hpi, hours post-infection

**Figure 4.** Gene expression of oxidative stress markers in RV-infected Vero and A549 cells. RT-qPCR was applied for analysis of gene expression of the oxidative stress markers heme oxygenase-1 (HMOX-1) and of the kappa light chain enhancer of B cells, subunit p65 (NF-kB/p65). As a positive control, oligomycin (30 µM) was applied to mock-infected controls at 48 hpi. Samples were collected at 72 hpi for RNA-extraction. Data are shown as percentages of the corresponding mock-infected controls and in the case of the positive control oligomycin as percentage of the untreated control. Gene expression was normalized against HUEL and HPRT-1. hpi, hours post-infection

**Figure 5.** Flow cytometric (A and C) and microscopic (B and D) analysis of reactive oxygen species (ROS) production in RV-infected Vero (A and B) and A549 (C and D) cells using the hydrogen peroxide sensitive probe DCF-DA. ROS levels were measured by flow cytometric analysis. Representative flow cytometry profiles of DCF fluorescence are shown for RV- and mock-infected Vero (A) and A549 cells (C) obtained at 3 hpi (hours post-infection). Oligomycin (30 µM)
or 0.03% H2O2 pretreated, mock-infected cells were included as controls. The marker M1 was set to include in the right portion of the flow cytometry histogram 50% of the total DCF-DA stained and untreated (mock) cell population. This marker was copied to histograms of oligomycin (30 µM) or 0.03% H2O2 pretreated or RV-infected cells. Additionally, values are given as the mean (± SE) of three independent experiments.

**Figure 6.** (A) and (B) mRNA expression analysis of mitochondrial biomarkers and (C) and (D) protein expression analysis of a complex II subunit in RV-infected Vero and A549 cells. RT-qPCR was applied for the analysis of the gene expression of (A) the nuclear transcription factors (NRF) 1 and 2, and of (B) nuclear encoded subunit NDUFB1 of complex I, subunit A and B (SDHA and SDHB) of complex II [succinate dehydrogenase (SDH)], subunit UQCRC1 of complex III, and COX4I1 of complex IV. Data are shown as percentages of the corresponding mock-infected controls. Gene expression was normalized against HUEL and HPRT-1. The mitochondrial protein expression level of SDHA was resolved by a 10% SDS-page using 70 µg of mitochondria isolated from RV- and mock-infected Vero (C) and A549 (D) cells. The blot was probed with SDHA and VDAC (loading control) antibodies.

**Figure 7.** Effect of RV infection on the mRNA and protein expression level of Mfn2 in A549 and Vero cells. (A) The transcript level of mitofusin 2 (Mfn2) was assessed by RT-qPCR. Data are shown as percentages of the corresponding mock-infected controls. Gene expression was normalized against HUEL and HPRT-1. The mitochondrial protein expression level of Mfn2 was resolved by a 10% SDS-page using 30 µg of mitochondria isolated from RV- and mock-infected A549 (B) and Vero (C) cells. The blot was probed with Mfn2 and VDAC3 (loading control) antibodies.
FIG 2
FIG 3
FIG 5
FIG 6
FIG 7
<table>
<thead>
<tr>
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<th>Sequence (5' - 3')</th>
<th>Temp (°C)</th>
<th>Ref</th>
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<td>(3)</td>
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<td></td>
<td>reverse: TGCTTTTTCACCAGCAAGCT</td>
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<td>(29)</td>
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<td></td>
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<td>63</td>
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hypoxanthine phosphoribosyltransferase 1 (HPRT1); solute carrier family 30 member 9 (HUEL); nuclear respiratory factor 1 and 2 (NRF-1 and -2); SDHA and B, succinate dehydrogenase, subunit A and B; heme oxygenase-1 (HMOX-1); kappa light chain enhancer of B cells subunit p65 (NF-kB/p65); mitofusin 2 (Mfn2)  

$^5$ designed using the ProbeFinder software (version 2.45, Roche)
Table 2. Summary of mitochondrial ETC enzyme activities in three different cell lines after RV-infection.

<table>
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<tr>
<th>Time line (dpi)</th>
<th>Cell</th>
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<th>Complex II</th>
<th>Complex III</th>
<th>Complex IV</th>
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<td>93 ±13 (n = 5)</td>
<td>108 ±16 (n = 3)</td>
<td>160 ±27 (n = 3)</td>
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<td>76 ±9 (n = 4)</td>
<td>84 ±15 (n = 5)</td>
<td>107 ±15 (n = 3)</td>
<td>108 ±18 (n = 3)</td>
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<td>Vero</td>
<td>241 ±45 (n = 4)</td>
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<td>231 ±31 (n = 3)</td>
<td>199 ±31 (n = 3)</td>
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<td>142 ±15 (n = 3)</td>
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<td>212 ±23 (n = 3)</td>
<td>144 ±17 (n = 4)</td>
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Values represent group means (± SE); n, sample size; dpi, days post-infection.