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**Mitochondrial and bioenergetic dysfunction in human hepatic cells infected with
dengue 2 virus**

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apoptosis, HepG2 cells

Summary

Dengue virus infection affects millions of people all over the world. Although the clinical manifestations of dengue virus-induced diseases are known, the physiopathological mechanisms involved in deteriorating cellular function are not yet understood. In this study we evaluated for the first time the associations between dengue virus-induced cell death and mitochondrial function in HepG2, a human hepatoma cell line. Dengue virus infection promoted changes in mitochondrial bioenergetics, such as an increase in cellular respiration and a decrease in $\Delta\Psi_m$. These alterations culminated in a 20% decrease in ATP content and a 15% decrease in the energy charge of virus-infected cells. Additionally, virus-infected cells showed several ultrastructural alterations, including mitochondria swelling and other morphological changes typical of the apoptotic process. The alterations in mitochondrial physiology and energy homeostasis preceded cell death. These results indicate that HepG2 cells infected with dengue virus are under metabolic stress and that mitochondrial dysfunction and alterations in cellular ATP balance may be related to the pathogenesis of dengue virus infection.

Introduction

Dengue virus (DV) infection affects 50-100 million people and it is considered the major human arbovirolosis [1-3]. Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), the more severe manifestations of DV infection, hospitalize and kill several hundred thousand people annually and represent a serious public health, economic and social problem, especially in tropical areas of the globe [1,2,4]. Although the clinical manifestations of DHF/DSS have already been described, the pathophysiology of this disease is still not completely understood, and the molecular mechanisms involved in organ and cellular dysfunction are not yet clear [2-4].

Several lines of evidence support the idea that liver dysfunction is a characteristic of severe dengue infection. Analyses of liver autopsies from individuals with DHF revealed extensive areas of tissue damage, with foci of necrosis, steatosis and apoptosis, characterized by the presence of apoptotic bodies [5,6]. Viral antigens were detected near the lesioned areas, suggesting an association between virus replication and hepatic damage [6]. Additionally, it has been reported that cytokines production is implicated in liver dysfunction [2,7].

Tissue injury is responsible for many of the clinical manifestations observed in DHF/DSS [5,6], although metabolic disturbances may also be involved [8,9]. In accordance with these hypotheses, it has been shown that DV infection induces apoptosis in different cell types in culture [10]. Some studies have demonstrated the involvement of anti- and pro-apoptotic proteins of the Bcl-2 family [9-11] and the participation of reactive oxygen species [12], suggesting that altered permeability of mitochondrial membranes is involved in the process of cell death following DV infection.

For a series of viruses, including flaviviruses, the association between virus-induced cell death and the integrity of mitochondrial membranes has been extensively studied [13-16]. Viral proteins that interact with mitochondria are able to modulate the permeability of mitochondrial membranes and the liberation of components from the inter-membrane space to cytoplasm and regulate the process of apoptosis [13-15]. In the case of pro-apoptotic viruses, the hallmark of cell damage is an abrupt decrease in mitochondrial membrane electrochemical potential ($\Delta\Psi_m$) [16].

Although it is well known that altered permeability states can compromise mitochondrial physiology, ATP synthesis and consequently cell function, the study of mitochondrial bioenergetic dysfunction during virus infection has not been characterized in detail. In the case of DV, the possibility that mitochondrial bioenergetics and cellular energy state are associated with the deterioration of cell function during the course of infection has never been addressed.

In the present work it is demonstrated for the first time that DV infection of a human hepatoma cell line (HepG2) leads to alterations in the bioenergetic function of mitochondria, indicated by a decrease in $\Delta\Psi_m$, altered respiratory properties and mitochondrial morphology. These events compromise cellular energy homeostasis and may be related to the pathophysiology of DV infection.

Materials and Methods

Cell culture and virus propagation

HepG2 cells were acquired from American Type Cell Collection (USA). HepG2 is a differentiated human hepatocellular carcinoma cell line, which preserves many of the morphological and functional characteristics of hepatocytes [17-19], thus representing an interesting model for the study of liver bioenergetics during DV infection.

Cells were cultured on appropriate medium (Minimum Essential Medium with 5mM glucose) supplemented with 10% fetal bovine serum (Invitrogen Corporation, USA), 100 U/mL penicillin, 100 g/mL streptomycin, 0.22% sodium bicarbonate and 0.2% HEPES, pH 7.4 in a CO₂ humid incubation chamber at 37°C. For fluorescence analyses, cells were grown on glass coverslips on a 24 well plate. For all other assays, cells were grown on plastic Petri dishes. In each assay, cells were seeded at a density of 10⁵ cell per mL. After 2 days, cells were either mock-infected or infected with DV serotype 2 Asiatic strain 16681 (m.o.i. of 1 plaque forming units per cell). Assays were performed 48 hours after infection, since peak of DV replication in HepG2 cells occurs during this period (data not shown). For preparations of virus stocks, DV serotype 2 Asiatic strain was propagated in C6/36 *A. albopictus* cells.

Oxygen Consumption

Oxygen consumption was measured in an oximeter (Yellow Springs Instruments Co., USA or Hansatech, UK) using a Clark type electrode, in a 1.0mL water jacket maintained under constant stirring, at 37°C. Intact and permeabilized HepG2 cells were used for analyses of respiratory properties of mitochondria.

For measurements of oxygen consumption of intact cells, approximately 5×10^6 cells were suspended in culture medium (MEM) without fetal bovine serum, as described elsewhere [20]. After recording basal respiration, 3µg/mL oligomycin was added to record oligomycin-independent oxygen consumption. Oxygen consumption in the presence of oligomycin represents the sum of proton leak through the inner mitochondrial membrane plus any non-mitochondrial oxygen consumption. The block of respiration observed after the addition of antimycin A reveals that the non-mitochondrial oxygen consumption in HepG2 cells is negligible (Fig. 1a). Maximum respiration was achieved by adding FCCP 200 nM. Optimum FCCP concentration was determined after FCCP titration (40-500 nM, data not shown).

For respiratory complexes activities, respiratory control ratio (RCR) and ADP/O ratio calculations, permeabilized HepG2 cells were used. In this case, cells were suspended in respiration media containing 0.25 M mannitol, 0.1% bovine serum albumin, 10 mM MgCl₂, 10 mM HEPES, 10 mM KH₂PO₄, pH 7.2, as described elsewhere [21]. Immediately after the addition of cells (approximately 5×10^6 cells), digitonin (0.005%, w/v) was added, followed by respiratory modulators and substrates. Complex I maximum activity was determined after the addition of 5 mM pyruvate, 5 mM malate and 5 mM glutamate and 1 mM ADP. For RCR analyses, 1 µM rotenone and 12 mM succinate were added. After

recording state 4 respiration, 0.1 mM ADP was added to induce state 3 respiration. Due to the presence of endogenous substrates on cell preparation, RCR, in each case, was calculated after the second addition of ADP. Following the first addition of ADP, endogenous adenylates are converted to ATP, an activator of succinate dehydrogenase [22,23] and respiratory control was clearly measured following the second addition of ADP. ADP/O ratios were calculated after each addition of ADP.

Mitochondrial membrane potential ($\Delta\Psi_m$)

JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; Molecular Probes) was used to measure changes in $\Delta\Psi_m$ following DV infection. JC-1 has the property to selectively enter into mitochondria and according to the magnitude of $\Delta\Psi_m$, its oligomeric state changes reversibly and so does its fluorescence properties. The ratio red:green fluorescence intensity of JC-1 gives an index of the $\Delta\Psi_m$, where the higher the ratio, the higher the $\Delta\Psi_m$ [24]. Cells were incubated with 5 $\mu\text{g/ml}$ JC-1 at 37 °C for 1 hour. Microscopic analyses of fluorescent images were performed using an epifluorescence microscope (Olympus BX40, Tokio, Japan) equipped with appropriate filters and using 40 \times oil-immersion objectives (Ex 485 nm; Em JC-1 Red 546, Em JC-1 Green 532 nm).

HPLC of cellular nucleotides

HepG2 nucleotides were analyzed by reverse-phase ion-pair high-performance liquid chromatography (HPLC) in a LC-10AS chromatograph with a high-pressure phase mixer and a UV-visible detector (SPD-10A; Shimadzu, Japan) operated at 254 nm as described in [25]. Briefly, cells were disrupted with liquid N₂ and cellular proteins were

precipitated with cold trichloroacetic acid (6%, w/v). Before injection, samples were neutralized with 2.5 M K₂CO₃. Samples and standards were eluted from the column (Hypersil ODS, Supelco Co.) with a linear solvent gradient between phase A (10 mM tetrabutylammonium, 10 mM KH₂PO₄, 0.25% methanol) and phase B (5.6 mM tetrabutylammonium, 50 mM KH₂PO₄, 30% methanol). Identities of AMP, ADP, and ATP peaks were confirmed by spiking of samples with nucleotides standards. Nucleotides were quantified by external calibration of peak areas of standards.

Energy charge (EC) was calculated according to the following equation, as described in reference [26]:

$$EC = [(ATP)+0.5 \times (ADP)] / [(AMP)+(ADP)+(ATP)] \quad (1)$$

Transmission electron microscopy

HepG2 cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Cells were post-fixed on 1% OsO₄ in cacodylate buffer plus 5 mM CaCl₂ and 0.8% potassium ferricyanide, dehydrated in acetone series and infiltrated in Epon. Polymerization was carried out for 72 h. Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL 1210 transmission electron microscope.

Statistical analysis

Statistical analyses were performed using Statgraphics version 4.0 (Statistical Graphics Corporation/Plus Ware). Results are presented as means (\pm SE). Paired and unpaired Student's t tests were used for comparison of mock-infected (control) and dengue virus-infected cells. Two-tailed *P* values < 0.05 were considered statistically significant.

Results

DV infection induces alterations in mitochondrial respiratory properties in HepG2 cells

The effects of DV infection on the respiratory properties of mitochondria was investigated in intact and permeabilized HepG2 cells. In order to study the effects of DV infection on cellular respiration under physiological conditions, intact HepG2 cells were used. Figure 1a shows a typical record of oxygen consumption in intact HepG2 cells where it can be seen that both basal and oligomycin-independent respiration are increased in DV-infected cells. Basal respiration significantly increased from $3.62 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ in mock-infected cells to $4.02 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ in DV-infected cells (Fig. 1b; $P < 0.05$). Additionally, oligomycin-independent respiration increased from $0.83 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ to $1.09 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ in infected HepG2 cells (Fig. 1c; $P < 0.05$). FCCP addition to either mock-infected and infected cells stimulated maximum respiration in a similar way (Fig. 1a), suggesting that DV infection did not cause any alteration in the electron chain capacity of HepG2 cells.

Figures 2a and 3a show typical records of oxygen consumption in mock-infected and DV-infected permeabilized cells using, respectively, NADH-linked or succinate as respiratory substrates. In agreement with the results obtained from intact cell, both basal and oligomycin-independent respiration was also increased in permeabilized cells (Fig. 2a). Basal respiration increased from $0.60 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ in mock-infected cells to $0.72 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ in DV-infected cells (Fig. 2b; $P < 0.05$), representing a 20% increase. There was no difference between Complex I respiration from mock-infected and infected HepG2 cells where oxygen consumption was, respectively, 1.54 and $1.61 \text{ nmol O}_2/10^6 \text{ cell} \times \text{min}^{-1}$ (Fig. 2a). Uncoupled or oligomycin-independent respiration raised from

0.22 nmol O₂/10⁶ cell × min⁻¹ to 0.37 nmol O₂/10⁶ cell × min⁻¹ in infected HepG2 (Fig. 2c; *P*<0.05), indicating that even in permeabilized HepG2 cells, DV infection induces changes in the respiratory properties of mitochondria by altering mitochondrial membrane permeability. In agreement with the results from intact cells, following FCCP addition maximum respiration did not differ between mock-infected and infected cells (Fig. 2a).

Since complex I-driven respiration was better accessed under maximum conditions (using 1 mM ADP), ADP/O ratio was determined feeding Complex II. Figure 3a shows that after the addition of succinate, a substrate of Complex II, the rate of oxygen consumption in both cases rose significantly. It is well established that when cells are maintained in an appropriated medium containing reducing substrates that can donate hydrogen atoms (electrons and protons) to the respiratory electron chain, mitochondria start to respire at a slow rate due to the build up of a maximum proton gradient (state 2 or state 4 respiration) [27]. Once ADP and Pi are added, respiration is increasingly stimulated due to ADP conversion to ATP by F₀F₁-ATP-synthase using the proton gradient established across the inner membrane by the electron transport chain (state 3 respiration) and ΔΨ_m is partially reduced. When limiting amounts of ADP are added to mitochondria, state 3 respiration persists until all ADP is converted to ATP and, at this point, the proton gradient is increased again and oxygen consumption is reduced [27]. Accordingly, figure 3a also shows that the rate of oxygen consumption in state 4 respiration was significantly higher in DV-infected cells. The calculated state 4 respiration rates were, respectively, 0.27 and 0.64 nmol O₂/10⁶ cell × min⁻¹ for mock-infected and DV-infected HepG2 cells (*P*<0.05). The rates of state 3 respiration did not differ significantly between the two samples being 2.01 for mock-infected and 2.31 nmol O₂/10⁶ cell × min⁻¹ for DV-infected cells. These results

are in agreement with the calculated rates of oxygen consumption using NADH-linked substrates (Fig. 2).

The RCR, which is indicative of the state of mitochondrial permeability, is shown in Figure 3b. The average RCR was 12.20 in mock-infected cells and 3.98 in virus-infected cells, suggesting that, even in permeabilized HepG2 cells, mitochondria had lost some of their natural intactness after DV infection. Figure 3c shows that the stoichiometry of the oxidative phosphorylation, ADP consumed per mol of oxygen atoms reduced to water (ADP/O ratio), was significantly decreased from 1.81 to 1.49 in DV-infected cells, indicating a decreased efficiency in ATP synthesis following infection.

The results with permeabilized HepG2 cells are in agreement with those obtained with intact cells. The increase in oligomycin-independent respiration (Figs. 1c and 2c) and in state 4 respiration (Fig. 3a) along with the decrease in ADP/O ratio (Fig. 3c) reinforce the idea that DV-infection affects mitochondrial membrane permeability and thus the efficiency of ATP synthesis in HepG2 cells.

DV infection induces a decrease in $\Delta\Psi_m$ in HepG2 cells

In order to better investigate the altered respiratory properties of HepG2 cells following DV infection, $\Delta\Psi_m$ was evaluated. Figure 4 shows fluorescence analyses of cells loaded with JC-1 dye. The red:green fluorescence intensity was 3-fold lower in virus-infected cells, suggesting that DV infection promoted a decrease in $\Delta\Psi_m$ (Fig. 4e). In addition, both red and green fluorescence intensities were decreased in virus-infected cells (Fig. 4c and 4d), indicating a decreased incorporation of JC-1 into mitochondria and possibly an altered permeability state of mitochondrial membranes.

DV infection affects ATP content and energy charge in HepG2 cells

Nucleotide content was analyzed to investigate whether the uncoupling of mitochondria and the decrease in $\Delta\Psi_m$ in DV-infected cells were interfering with ATP synthesis. The average amounts of AMP, ADP and ATP in mock-infected and virus-infected samples were, respectively, 0.3 and 1.7 nmols/ 10^6 cells; 2.6 and 5.0 nmols/ 10^6 cells; 18.7 and 15.5 nmols/ 10^6 cells (Fig. 5a). These differences corresponded to a 5-fold and a 2-fold increase in AMP and ADP levels, respectively, and a 20% decrease in ATP levels in virus-infected cells. All differences shown are significant ($P<0.05$).

The cellular adenylate pool composed of ATP, ADP and AMP includes the majority of the available chemical energy in cellular metabolism [26]. From the results shown in Figure 4a, the total nucleotide pool was 22.5 nmols nucleotide/ 10^6 cells for mock-infected cells and 22.1 nmols nucleotide/ 10^6 cells for virus-infected cells (Fig. 5b). This result shows that infection did not alter the total amount of nucleotides, but instead their distribution. This altered distribution resulted in a decrease in the energy charge (EC) reflects the cellular state of free energy availability. An EC value of 1 indicates that all the available energy is present as ATP and an EC value of zero means that only AMP is present [26]. The calculated EC values were 0.93 and 0.81, respectively, for mock-infected and virus-infected cells (Fig. 5c), indicating that infection promoted a significant decrease ($P<0.005$) in this index.

DV infection induces ultrastructural alterations in HepG2 cells

Transmission electron microscopy analyses were performed to associate the alterations in mitochondrial bioenergetics with possible morphological changes. As shown

in Figure 6, mock-infected cells exhibit mitochondria with characteristic electron-dense matrix (Fig. 6a; arrows), contrasting to swollen mitochondria with a loss of electron density in the matrix of DV-infected cells (Fig 6b; arrows). In a total of 164 mitochondria counted in mock-infected cells, 95% presented normal ultrastructure. In DV-infected cells, 144 mitochondria were counted and 75% showed some degree of morphological alterations. In addition to the morphological changes in mitochondria, other ultrastructural alterations following DV infection can be pointed out in Figure 7, including the formation of membrane blebs (Fig. 7c and 7d; arrow heads) and the localization of mitochondria around the nuclear membrane (Fig. 7c; arrows). Mock-infected cell, on the other hand, exhibit an intact cellular membrane (Fig. 7a). Electron dense particles seen in Figure 7b are compatible with DV particles [30].

Discussion

Virus infections elicit a number of cellular responses in the host that may create a hostile environment to the virus and therefore to control replication and minimize the deleterious effects of infection. Viruses, on the other hand, have developed strategies to curtail the cellular responses and ensure the success of infection. Among different hosts and viruses many of these responses involve alterations in energy metabolism, in particular an increased utilization of glucose molecules, alterations in the production of ATP and the modulation of mitochondrial functions [13,31-33]. This last response is evident concerning the process of apoptosis, which most frequently includes alterations in mitochondrial integrity [13,14,32]. The present work shows that cells infected with DV present mitochondrial dysfunction characterized by functional and ultrastructural alterations.

The respiratory properties of HepG2 cells were clearly altered by DV infection, which promoted an increase in cellular oxygen consumption along with a decrease in RCR and ADP/O (Figs. 1a, 1b; Figs. 2a, 2b and Fig. 3). A decrease in the RCR suggests that significant morphologic alterations of mitochondria may be undertaken in HepG2 cells after DV infection, which can be confirmed from the TEM analyses (Fig. 6). In addition, the decrease in ADP/O ratio (Fig. 3c) and the increase in both oligomycin-independent respiration (Figs. 1c and 2c) and state 4 respiration (Fig. 3a) indicate that DV infection causes an uncoupling of the electron transfer chain from the phosphorylation of ADP, which results in a decreased efficiency for synthesis of ATP [34]. Both the decrease in RCR and in the ADP/O in virus-infected cells are due to the marked increase in the rate of state 4 respiration, since state 3 respiration was not significantly changed neither in Complex I nor Complex II-linked substrates (Figs. 2a and 3a). Additionally, the increase in oligomycin-

independent respiration also indicates an increase in proton leak, a feature of uncoupled oxidative phosphorylation in DV-infected cells (Figs. 1a, 1c and Figs. 2a and 2c).

Mitochondrial uncoupling reflects a flux of protons from the inter-membrane space to the mitochondrial matrix through channels other than the F_0F_1 ATP synthase and, therefore, not coupled to ATP synthesis. When mitochondrial uncoupling is physiological, there is an increase in electron transport and in oxygen consumption due to a small decrease in the $\Delta\Psi_m$ in order to maintain the electrochemical proton gradient and the synthesis of cellular ATP [34]. However, when mitochondrial uncoupling is important, one may expect a substantial decrease in $\Delta\Psi_m$, as observed in Figure 4, with several consequences for cellular energy homeostasis, as occurred with HepG2 cells infected with DV. The decrease in $\Delta\Psi_m$ in intact cells infected with DV may be explained, in part, by an increase in proton leak, in accordance with the increase in oligomycin-independent respiration (Fig. 1c).

The effects of DV infection on the respiratory properties and mitochondrial membrane depolarization, which decreased the efficiency of ATP synthesis, also promoted a significant decrease in the steady-state ATP concentration of HepG2 cells (Fig. 5a). This decrease in the content of cellular ATP may indicate that energy demand is increased due to the synthesis of viral proteins. By this way, it can be speculated that DV infection also affects cellular regulatory mechanisms that compensate for mitochondrial inefficiency, such as an increased production of glycolytic ATP, as observed in various situations, like alphavirus infection [33].

As shown in Figure 5a, although the total nucleotide pool was not altered (Fig. 5b), infection induced a significant change in the relative amounts of ATP, ADP and AMP. This led to a decrease in EC in virus-infected cells (Fig. 5c). EC in hepatocytes is usually around

0.9 [35], a value similar to that observed for mock-infected cells in the present work. In liver cells, when ATP synthesis is impaired, the EC may reach extremely low levels such as 0.7 [36]. Thus, the EC of 0.8 observed in virus-infected cells indicates a metabolic stress. It has been shown that myocarditis induced by CoxsackieB3 virus promoted a decrease in cellular EC, and it was suggested that this alteration in energy balance is involved in the pathogenesis of the Coxsackie virus infection [37]. Although the role of energy homeostasis in DV infection has never been addressed, it can be suggested that the deregulation of processes involved in ATP balance may be associated with the pathogenesis of DV infection.

The altered bioenergetic status caused by DV infection may be regarded as an initial sign of a more serious condition. Although cell viability was not altered in DV-infected cells in all m.o.i. used (data not shown), the small but significant decrease in cellular ATP and EC might be signaling an apoptotic stimulus, since it has been demonstrated that a transient decrease in the content of cellular ATP preceded the initiation of the apoptotic cascade in another cell type [38]. In agreement with these observations are the ultrastructural alterations in mitochondria in DV-infected cells (Fig. 6b), showing a loss of electron density in the matrix and mitochondrial swelling. This feature is characteristic of the permeability transition state, and, therefore, may indicate an increase in outer mitochondrial membrane permeability [16,39]. This association between mitochondrial structure and metabolic function has already been studied [40], however not in cells infected with DV. Although nuclear alterations typical of the apoptotic process have already been described in HepG2 cells infected with DV [8,41], this is the first report of mitochondrial morphologic alterations. Additionally, other apoptotic features can be

observed in DV-infected cells, such as the localization of mitochondria around the nucleus and membrane blebs (Figs. 7c and 7d), as previously shown for other cell types infected with DV [8,10,42]. Accordingly, Hoechst fluorescence indicated an increase in the percentage of apoptotic cells from 5-10% in mock-infected cells to 15-20% in DV infected cells (data not shown).

The mechanisms through which DV infection most contribute to the observed functional and morphological alterations remain open. Cellular proteins that interact directly with mitochondria, such as pro- or anti-apoptotic Bcl-2 family proteins, might be involved [8,10,13,43]. Additionally, reactive oxygen and nitrogen species may contribute to mitochondrial dysfunction, since DV infection increases oxidative stress both *in vivo* and *in vitro* [12,44,45].

Specific viral proteins, which interact with mitochondria, have the potential to affect mitochondrial membrane permeability and thus the process of apoptosis, as shown for other viruses [13-15]. Our observations that ADP/O ratio is decreased and oligomycin-independent respiration is increased after infection favor the hypothesis that viral proteins or viral sub-products could be interacting with mitochondrial membranes of HepG2 cells, altering their permeability, increasing proton leak and thus causing the described alterations in mitochondrial physiology. Indeed, further studies are needed to clarify the causal link between mitochondrial physiology and the apoptotic events in DV infection. It is important to mention that the fact that either basal and oligomycin-independent respiration are stimulated by DV infection rules out, at least at this stage of DV infection, an important impairment of respiratory electron chain complexes activities, as reported in some

mitochondrial dysfunction as observed in sepsis in both human [28] and animal [29] studies.

In the present work we described for the first time functional and structural alterations in mitochondria, related to bioenergetics and energy homeostasis of cultured human hepatic cells infected with DV. These results contribute to the understanding of the events occurring in host cells during the course of DV infection and possibly to the processes leading to cell death. It seems that derangements in energy metabolism are associated with apoptosis, and that this mechanism of cell death is intimately associated with the pathogenesis of DHF/DSS [10]. Our results indicate that derangements in energy metabolism are signs of DV infection, which seem to precede cell death, since cell viability, in the time course observed, was not altered. In conclusion, the identification of early signs of metabolic deterioration could be a valuable tool to develop strategies to minimize cell damage and ultimately interfere with the replication cycle of DV.

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References

- [1]D.J. Gubler, The global emergence/resurgence of arboviral diseases as public health problems, *Arch. Med. Res.* 33 (2002) 330-342.
- [2]D.J. Gubler, Dengue and dengue hemorrhagic fever, *Clin. Microbiol. Rev.* 11 (1998) 480–496.
- [3]J.S. Mackenzie, D.J. Gubler, L.R. Petersen, Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses, *Nat. Med.* 10 (2004) S98-109.
- [4]M.G. Gúzman, G. Kouri, Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges, *J. Clin. Virol.* 27 (2003) 1–13.
- [5]A. Couvelard, P. Marianneau, C. Bedel, M.T. Drouet, F. Vachon, D. Henin, V. Deubel, Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis, *Hum. Pathol.* 30 (1999) 1106-1110.
- [6]M.R. Huerre, N. T. Lan, P. Marianneau, N. B. Hue, H. Khun, N. T. Hung, N. T. Khen, M. T. Drouet, V. T. Huong, D. Q. Ha, Y. Buisson, V. Deubel, Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children, *Virchows Arch.* 438 (2001) 107-115.
- [7]U.C. Chaturvedi, R. Agarwal, E.A. Elbishbishi, A.S. Mustafa, Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis, *FEMS Immunol. Med. Microbiol.* 28 (2000) 183-188.

- [8]P. Marianneau, M. Flamand, V. Deubel, P. Després, Apoptotic cell death in response to dengue virus infection: the pathogenesis of dengue haemorrhagic fever revisited, *Clin. Diagn. Virol.* 10 (1998) 113-119.
- [9]A. Catteau, G. Roue, V. J. Yuste, S. A. Susin, P. Despres, Expression of dengue ApoptoM sequence results in disruption of mitochondrial potential and caspase activation, *Biochimie* 85 (2003) 789–793.
- [10]M.P. Courageot, A. Catteau, P. Desprès, Mechanisms of dengue virus-induced cell death, *Adv. Virus Res.* 60 (2003) 157-186.
- [11]A. Catteau, O. Kalinina, M.C. Wagner, V. Deubel, M.P. Courageot, P. Desprès, Dengue virus M protein contains a proapoptotic sequence referred to as ApoptoM, *J. Gen. Virol.* 84 (2003) 2781–2793.
- [12]Y.L. Lin, C. C. Liu, J. I. Chuang, H. Y. Lei, T. M. Yeh, Y. S. Lin, Y. H. Huang, H. S. Liu, Involvement of oxidative stress, NF-IL-6, and RANTES expression in dengue-2-virus-infected human liver cells, *Virology* 276 (2000) 114-126.
- [13]H. Everett, G. McFadden, Viruses and Apoptosis: Meddling with Mitochondria, *Virology* 288 (2001) 1-7.
- [14]F. Verrier, B. Mignotte, G. Jan, C. Brenner, Study of PTPC composition during apoptosis for identification of viral protein target, *Ann. NY Acad. Sci.* 1010 (2003) 126-142.
- [15]C. Piccoli, R. Scrima, A. D'Aprile, M. Ripoli, L. Lecce, D. Boffoli, N. Capitano, Mitochondrial dysfunction in hepatitis C virus infection, *Biochim. Biophys. Acta* 1757 (2006) 1429-1437.

- [16]G. Kroemer, J.C. Reed, Mitochondrial control of cell death, *Nat. Med.* 6 (2000) 513-519.
- [17]Q. Chen, Y. Xia, Z. Qiu, Effect of eadysterone on glucose metabolism in vitro, *Life Sci.* 78 (2006) 1108-1113.
- [18]B.B. Knowles, C.C. Home, D.P. Aden, Human Hepatocellular carcinoma cell line secrete major proteins and hepatitis B surface antigens, *Science* 209 (1980) 497-499.
- [19]A. Kosaki, N.J.G. Webster, Effect of dexamethasone on the alternative splicing of the insulin receptor mRNA and insulin action in HepG2 hepatoma cells, *J. Biol. Chem.* 29 (1993) 21990-21996.
- [20]E. Hutter, K. Renner, G. Pfister, P. Stockl, P. Jansen-Durr, E. Gnaiger, Senescence-associated changes in respiration and oxidative phosphorylation in primary human fibroblasts, *Biochem. J.* 380 (2004) 919–928.
- [21]K. Sakurai, A.I. Cederbaum, Oxidative stress and cytotoxicity induced by ferric-nitrosylacetate in HepG2 cells that express cytochrome P450 2E1, *Mol. Pharmacol.* 54 (1998) 1024-1035.
- [22]M. Gutman, E.B. Kearney, T.P. Singer, Control of succinate dehydrogenase in mitochondria, *Biochemistry* 10 (1971) 4763-4770.
- [23]C. Bhuvaneshwaran, C.H. Ho, C.L. Wadkins, Inactivation of coupled respiration of mitochondria by inorganic arsenate and partial restoration by ATP, *Biochem. Biophys. Res. Commun.* 49 (1972) 690-697.
- [24]M. Reers, T.W. Smith, L.B. Chen, J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential, *Biochemistry* 30 (1991) 4480-4486.

- [25]D. Huang, Y. Zhang, X. Chen, Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by high-performance liquid chromatography, *J. Chrom. B* 784 (2003) 101-109.
- [26]D.E. Atkinson, *Cellular energy metabolism and its regulation*, Academic Press, New York, 1977.
- [27]B. Chance, G.R. William, The respiratory chain and oxidative phosphorylation, *Adv. Enzymol. Relat. Subj. Biochem.* 17 (1956) 65-134.
- [28]A.D. Svistunenko, N. Davies, D. Brealey, M. Singer, C.E. Cooper, Mitochondrial dysfunction in patients with severe sepsis: an EPR interrogation of individual respiratory chain components, *Biochim. Biophys. Acta* 1757 (2006) 262-272.
- [29]D. Brealey, S. Karyampudi, T.S. Jacques, M. Novelli, R. Stidwill, V. Taylor, R.T. Smolenski, M. Singer, Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286(2004) R491-497.
- [30]O. Barth, Replication of dengue viruses in mosquito cell cultures-a model from ultrastructural observations, *Mem. Inst. Oswaldo Cruz.* 87 (1992) 565-574.
- [31]L.M. Romero, K. M. Raley-Susman, D. M. Redish, S. M. Brooke, H. C. Horner, R. M. Sapolsky, Possible mechanism by which stress accelerates growth of virally derived tumors, *Proc. Natl. Acad. Sci. USA.* 89 (1992) 11084-11087.
- [32]H. Everett, G. McFadden, Apoptosis: an innate immune response to virus infection, *Trends Microbiol.* 160 (1999) 160-5.

- [33]T. El-Bacha, M.M. Menezes, M.C. Azevedo e Silva, M. Sola-Penna, A.T. Da Poian, Mayaro virus infection alters glucose metabolism in cultured cells through activation of the enzyme 6-phosphofructo 1-kinase, *Mol. Cell. Biochem.* 266 (2004) 191-198.
- [34]G.C. Brown, P.L. Lakin-Thomas, M.D. Brand, Control of respiration and oxidative phosphorylation in isolated rat liver cells, *Eur. J. Biochem.* 192 (1990) 355-362.
- [35]D.E. Atkinson, G.M. Walton, Adenosine triphosphate conservation in metabolic regulation, *J. Biol. Chem.* 242 (1967) 3239-3241.
- [36]K.O. Raivio, M.P. Kekomaki, P.H. Maenpaa, Depletion of liver adenine nucleotides induced by D-fructose, Dose-dependence and specificity of the fructose effect. *Biochem. Pharmacol.* 18 (1969) 2615-2624.
- [37]A.Waldenstrom, J. Fohlman, N. G. Ilback, G. Ronquist, R. Hallgren, B. Gerdin, Coxsackie B3 myocarditis induces a decrease in energy charge and accumulation of hyaluronan in the mouse heart, *Eur. J. Clin. Invest.* 23 (1993) 277-282.
- [38]D.S. Izyumov, A.V. Avetisyan, O.Yu Pletjushkina, D.V. Sakharov, K.W. Wirtz, B.V. Chernyak, V.P. Skulachev, "Wages of Fear": transient threefold decrease in intracellular ATP level imposes apoptosis, *Biochim. Biophys. Acta* 1658 (2004) 141-147.
- [39]M. Crompton, The mitochondrial permeability transition pore and its role in cell death, *Biochem. J.* 341 (1999) 233-249.
- [40]E. Gottlieb, S.M. Armour, C.B. Thompson, Mitochondrial respiratory control is lost during growth factor deprivation, *Proc. Natl. Acad. Sci. U S A.* 99 (2002) 12801-12806.

- [41]P. Marianneau, A. M. Steffan, C. Royer, M. T. Drouet, A. Kirn, V. Deubel, Differing infection patterns of dengue and yellow fever viruses in a human hepatoma cell line, *J. Infect. Dis.* 178 (1998) 1270-1278.
- [42]J.A. Mosquera, J. P. Hernandez, N. Valero, L. M. Espina, G. J. Anez, Ultrastructural studies on dengue virus type 2 infection of cultured human monocytes, *Virology* 2 (2005) 26.
- [43]C.J. Lee, C.L. Liao, Y.L. Lin, Flavivirus activates phosphatidylinositol 3-kinase signaling to block caspase-dependent apoptotic cell death at the early stage of virus infection, *J. Virol.* 79 (2005) 8388-8399.
- [44]N. Valero, L. M. Espina, G. Anez, E. Torres, J. A. Mosquera, Short report: increased level of serum nitric oxide in patients with dengue, *Am. J. Trop. Med. Hyg.* 66 (2002) 762-764.
- [45]L. Gil, G. Martinez, R. Tapanes, O. Castro, D. Gonzalez, L. Bernardo, S. Vazquez, G. Kouri and M. G. Gúzman, Oxidative stress in adult dengue patients, *Am. J. Trop. Med. Hyg.* 71 (2004) 652-657.

Figure legends

Figure 1: Dengue virus infection increases oxygen consumption and alters respiratory properties of intact HepG2 cells. Cells were either mock-infected (black trace and bar) or DV-infected (gray trace and bar) and oxygen consumption was measured on culture medium without fetal bovine serum. (a) Typical record of oxygen consumption in intact cells. (b) Basal respiration. (c) Oligomycin-independent respiration. Values correspond to the average of 5 independent experiments. *significantly different from mock-infected cells (Paired t-test; $P < 0.05$).

Figure 2: Dengue virus infection increases oxygen consumption and alters respiratory properties of permeabilized HepG2 cells. Cells were either mock-infected (black trace and bar) or DV-infected (gray trace and bar) and oxygen consumption was measured on respiration medium. (a) Typical record of oxygen consumption in permeabilized cells following the addition of 5 mM pyruvate + 5 mM malate + 5 mM glutamate and 1 mM ADP. (b) Basal respiration. (c) Oligomycin-independent respiration. Values correspond to the average of 6 independent experiments. *significantly different from mock-infected cells (Paired t-test; $P < 0.05$).

Figure 3: Dengue virus infection increases oxygen consumption and decreases RCR and ADP/O ratio of permeabilized HepG2 cells. Cells were either mock-infected (black trace and bar) or DV-infected (gray trace and bar) and oxygen consumption was measured on respiration medium. (a) Typical record of oxygen consumption in permeabilized cells following the addition of 12 mM succinate and 0.1 mM ADP. (b) Respiratory control ratio (state 3/state 4 oxygen consumption rate). (c) ADP/O ratio. Values correspond to the

average of 5 independent experiments. *significantly different from mock-infected cells (t-test; $P < 0.05$).

Figure 4: Dengue virus infection promotes a decrease in mitochondrial membrane potential of HepG2 cells. Cells were incubated with 5 $\mu\text{g/ml}$ of the dye JC-1 for 1 h at 37°C. JC-1 fluorescence in mock-infected (a, b) and in DV-infected cells (c, d) was captured using filters appropriate for red (a, c) and green (b, d) emission. (e) Red:green fluorescence intensity in mock-infected (black bar) and DV-infected cells (gray bar).

Figure 5: Dengue virus infection affects nucleotide content and energy charge of HepG2 cells. AMP, ADP and ATP contents were measured by reverse-phase ion-pair HPLC in mock-infected (black) or DV-infected (gray) HepG2 cells. (a) AMP, ADP and ATP contents. (b) Nucleotide pool (AMP+ADP+ATP). (c) Energy charge (EC), calculated from the equation $EC = [(ATP) + 0.5 \times (ADP)] / [(AMP) + (ADP) + (ATP)]$. Values correspond to the average of 6 independent experiments. *significantly different from mock-infected cells (t-test; $P < 0.05$).

Figure 6: Dengue virus infection promotes morphological alterations of mitochondria in HepG2 cells. (a) Transmission electron microscopy of mock-infected cell showing mitochondria with characteristic electron-dense matrix (arrows); (b) Transmission electron microscopy of DV-infected cell presenting swollen mitochondria with loss of electron density in the matrix (arrows). Bar= 500nm.

Figure 7: Dengue virus infection induces ultrastructural alterations in HepG2 cells.

(a) Mock-infected cell, presenting an intact cellular membrane; (b) Electron dense particles of DV-infected cell (arrows); (c, d) DV-infected cell showing membrane blebs (arrowheads) and mitochondria around the nucleus (arrows), N: nucleus. Bar=500nm.

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