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Cellular bioenergetics, caspase activity and glutathione in murine lungs infected with influenza A virus

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ABSTRACT

Inhibition of cellular respiration, oxidation of glutathione and induction of apoptosis have been reported in epithelial cells infected *in vitro* with influenza A virus (IAV). Here, the same biomarkers were investigated *in vivo* by assessing the lungs of BALB/c mice infected with IAV. Cellular respiration declined on day 3 and recovered on day 7 post-infection. For days 3–5, the rate (mean ± SD) of respiration ($\mu M\ O_2\ min^{-1}\ mg^{-1}$) in uninfected lungs was 0.103 ± 0.021 ($n=4$) and in infected lungs was 0.076 ± 0.025 ($n=4$, $p=0.026$). Relative cellular ATP (infected/uninfected) was 4.7 on day 2 and 1.07 on day 7. Intracellular caspase activity peaked on day 7. Cellular glutathione decreased by ≥10% on days 3–7. Lung pathology was prominent on day 3 and caspase-3 labeling was prominent on day 5. IAV infection was associated with suppression of cellular respiration, diminished glutathione, and induction of apoptosis. These functional biomarkers were associated with structural changes noted in infected mice.

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Introduction

Influenza A virus (IAV) inhibits cellular respiration (mitochondrial O_2 consumption), diminishes cellular glutathione (GSH) and triggers apoptosis (Chen et al., 2001; Derakhshan et al., 2006; Gibbs et al., 2003; Hennet et al., 1992; Nencioni et al., 2003; Yamada et al., 2004). For example, Madin-Darby canine kidney epithelial cells infected with influenza A/PR/8/34 exhibit a lower rate of mitochondrial O_2 consumption (Derakhshan et al., 2006). The virus protein PB1-F2 associates with the mitochondrial inner membrane (Gibbs et al., 2003; Yamada et al., 2004). Infection in mice reduces lung tissue GSH and inhibition of GSH impairs viral replication (Hennet et al., 1992; Nencioni et al., 2003). These processes are associated with the induction of apoptosis (Chen et al., 2001).

Other viruses are also known to disturb host-cell mitochondria. For example, simian virus 40-infected CV-1 cells (prepared from renal tissue of the African green monkey *Cercopithecus aethiops*) demonstrate reduced mitochondrial respiration (Norkin, 1977). Human herpes virus 1 and poliovirus inhibit host-cell respiration without affecting cellular ATP (Derakhshan et al., 2006; Koundouris et al., 2000). Thus, these viruses potently modulate mitochondrial functions in support of their survival (Arnoult et al., 2009; Boya et al., 2001; El-Bacha and Da Poian, 2013).

Induction of apoptosis requires mitochondria to leak cytochrome c and other small molecular weight pro-apoptotic molecules from the intermembrane mitochondrial space to the cytosol (Green and Kroemer, 2004). In the cytosol, cytochrome c binds to the apoptotic protease activating factor-1 (Apaf-1), triggering the caspase (cysteine-dependent aspartate-directed protease) cascade. Caspase activation provokes mitochondrial perturbations, which involve opening the permeability transition pores and collapsing the electrochemical potential. Thus, induction of apoptosis is linked to mitochondrial dysfunction (Tao et al., 2007).

Caspase-3 and other caspases are involved in proteolysis, cleaving at the second aspartate in the asp–glu–val–asp sequence (Talanian et al., 1997). Hence, the synthetic substrate *N*-acetyl-asp-glu-val-asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) can be

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used to monitor intracellular caspase activities. The released fluorogenic moiety AMC can be separated on HPLC and detected with a great accuracy (Alsuwaidi et al., 2013a, 2013b).

The term "cellular bioenergetics" refers to biochemical reactions involved in energy metabolism and the term "cellular respiration" (mitochondrial oxygen consumption) describes delivery of metabolites and O_2 to the mitochondria, oxidation of reduced metabolic fuels with passage of electrons to O_2, and synthesis of ATP. Altered tissue mitochondrial O_2 consumption, thus, reveals disturbances in these processes.

Measuring cellular respiration, using the principle of "O_2 quenching the phosphorescence of palladium II-*meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin" has been reported (Al Samri et al., 2011; Alsuwaidi et al., 2013a, 2013b). This analytical tool allows *in vitro* monitoring of cellular respiration over several hours. This biomarker, as well as cellular ATP, caspase activity, GSH and histology are investigated here in murine lungs infected with IAV. The primary objective of the study was to assess lung cellular bioenergetics after influenza infection using a well-established mouse model. The secondary objectives were to explore biomarkers that could predict the severity of influenza infections. The results show that IAV infection is associated with suppression of cellular respiration, diminished cellular GSH and induction of apoptosis.

Results

Lung bioenergetics, caspase activity, IFN-γ and histology after IAV infection of BALB/c mice

Fig. 1 shows a representative experiment of cellular bioenergetics and caspase activity on days 2, 3, 5 and 7 post-infection of BALB/c mice with a sub-lethal dose of IAV (286 $TCID_{50}$) as compared with mock-infected controls. O_2 consumption runs on day 3 are shown in Fig. 1a. The rate of respiration (k_c, in $\mu M\ O_2\ min^{-1}\ mg^{-1}$) in uninfected lungs was 0.13 and in infected lungs was 0.09 (30% lower). Respiration was inhibited by cyanide, a specific poison of cytochrome oxidase, confirming that oxidation occurred in the mitochondrial respiratory chain. The addition of glucose oxidase, catalyzes the reaction of D-glucose+O_2 to D-glucono-δ-lactone+H_2O_2, depleted the remaining O_2, confirming that cyanide inhibition occurred despite available dissolved O_2. Fig. 1b shows the values of k_c as a function of days post-infection; cellular respiration declined on day 3 and recovered on day 7. The values of k_c for uninfected lungs on days 3–5 were 0.103 ± 0.021 ($n=4$). The corresponding values for infected lungs were 0.076 ± 0.025 (26% lower, $n=4$), p-value$=0.026$. Thus, O_2 consumption was transiently repressed in the lungs infected with IAV. Cellular ATP, on the other hand, increased by 4.7-fold on day 2 in infected lungs and returned to the value of uninfected lungs on day 7 (Fig. 1b). The values of ATP for uninfected lungs on days 2 and 3 were 73 ± 28 pmol mg^{-1} ($n=3$). The corresponding values for infected lungs were 147 ± 46 ($n=3$), p-value$=0.100$. Thus, the initial burst of cellular ATP was not sustained, perhaps due to depressed respiration. These results confirm previous reports, showing a transiently depressed respiration and normal cellular ATP in epithelial cells infected with IAV (Derakhshan et al., 2006).

Representative HPLC runs for Ac-DEVD-AMC cleavage by the recombinant human caspase-3 are shown in Fig. 1c and d. The caspase-3 substrate Ac DEVD-AMC was detected by absorbance at 380 nm with a retention time of 3.5 min (Fig. 1c). The product AMC, on the other hand, was detected by fluorescence (380 nm excitation and 460 nm emission) with a retention time of ~5 min (Fig. 1d). The cleavage reaction was inhibited by zVAD-fmk (Fig. 1d). Caspase activity was set as the AMC peak area without zVAD-fmk minus the AMC peak area with zVAD-fmk.

Fig. 1e and f shows representative intracellular caspase activity on day 7. Caspase activity (arbitrary unit $mg^{-1}/10^3$) on day 7 in the uninfected lung was 51 and in the infected lung was 230 (a 4.5-fold increase). Caspase activities (infected/uninfected) on days 3 and 5 increased 2.5-fold and 3.5-fold, respectively (data not shown). The caspase activity for uninfected lungs on days 2–7 were 12 ± 19 ($n=6$). The corresponding values for infected lungs were 62 ± 85 ($n=6$), p-value$=0.093$. Thus, lungs infected with IAV demonstrated an increase in intracellular caspase activity.

Fig. 2 shows histological sections and caspase-3 labeling of the experiment in Fig. 1. As expected, uninfected lungs exhibited normal pulmonary parenchyma architecture and no evidence of inflammation (Fig. 2a). On days 2 and 3 post-infection, the histology revealed preserved pulmonary parenchyma, patchy mild interstitial thickening composed of a few inflammatory cells (predominantly lymphocytes) and vascular congestion within IAV-infected lungs. There were a few intra-alveolar macrophages. The infiltrate was more prominent on day 3 than day 2. Peribronchial inflammation was absent and caspase-3 labeling was negative on days 2 and 3 (Fig. 2b). On day 5, the histology revealed preserved pulmonary parenchyma, vascular congestion, prominent type-II pneumocytes and patchy moderate interstitial inflammatory infiltrates composed of lymphocytes and histiocytes. Caspase-3 labeling was 1% (Fig. 2b). Inflammation decreased by day 7 and caspase-3 labeling was negative (Fig. 2b). Thus, the height of disease was on days 3–5 post-infection with evidence of recovery by day 7.

Higher viral inoculums were subsequently tested. BALB/c mice were inoculated on day 0 with 50 μL PBS (Fig. 3a), 50 μL of 10,000 $TCID_{50}$ IAV (a sub-lethal dose, Fig. 3b), or 50 μL of 2500 $TCID_{50}$ IAV (a sub-lethal dose, Fig. 3c). The lungs were studied on days 3 and 6 post-inoculation. In uninfected lungs, there were intact alveoli and airspaces with minimal inflammation and mild focal vascular congestion; caspase-3 labeling was negative (Fig. 3a). With 10,000 $TCID_{50}$ IAV, lung histology on day 3 revealed mild-to-moderate acute bronchopneumonia with patchy interstitial and peribronchial mononuclear inflammatory cell infiltrate. Caspase-3 labeling was ~5%, lung tissue respiration decreased by ~30%, ATP increased by ~100% and IFN-γ increased by ~30% (Fig. 3b). On day 6, there was mild diffuse interstitial mononuclear inflammatory cell infiltrate. There were no hyaline membranes or edema, and the caspase-3 labeling was ~1%. Lung tissue respiration decreased by 10%, ATP increased by 33%, and IFN-γ increased by 200% (Fig. 3b). In Fig. 3c (infected with 2500 $TCID_{50}$), on day 3, there were severe patchy interstitial and bronchial mononuclear inflammatory cell infiltrates. Lymphocytes, plasma cells and macrophages expanded alveolar septae and focally filled alveolar spaces. Numerous apoptotic debris and focal alveolar edema were noted; caspase-3 labeling was ~10%. These findings were consistent with acute bronchopneumonia. On day 6, there were milder patchy interstitial and bronchial mononuclear inflammatory cell infiltrates. Apoptotic debris was present and caspase-3 labeling was ~1% (Fig. 3c). Thus, higher viral concentrations produced more severe disease. Consistently, cellular respiration on day 3 post-infection declined exponentially with IAV concentration and recovered on day 6 (Fig. 1S, Supplementary material). In a mouse inoculated with 100 μL of $10^{8.1}$ $TCID_{50}$ IAV (a lethal dose), the body weight also decreased exponentially as a function of days post-infection. The mouse died on day 10 post-infection (Fig. 2S, Supplementary material).

Lung GSH after IAV infection

Fig. 4 shows lung tissue GSH in uninfected and infected BALB/c mice. The mice were inoculated on day 0 with 50 μL PBS, 50 μL of 286 $TCID_{50}$ IAV, or 33 μL of $10^{8.1}$ $TCID_{50}$ IAV. The lungs were

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