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The respiratory syncytial virus (RSV) nonstructural proteins mediate RSV suppression of glucocorticoid receptor transactivation

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ABSTRACT

Respiratory syncytial virus (RSV)-induced bronchiolitis in infants is not responsive to glucocorticoids. We have shown that RSV infection impairs glucocorticoid receptor (GR) function. In this study, we have investigated the mechanism by which RSV impairs GR function. We have shown that RSV repression of GR-induced transactivation is not mediated through a soluble autocrine factor. Knock-down of mitochondrial antiviral signaling protein (MAVS), but not retinoic acid-inducible gene 1 (RIG-I) or myeloid differentiation primary response gene 88 (MyD88), impairs GR-mediated gene activation even in mock-infected cells. Over-expression of the RSV nonstructural protein NS1, but not NS2, impairs glucocorticoid-induced transactivation and viruses deleted in NS1 and/or NS2 are unable to repress glucocorticoid-induction of the known GR regulated gene glucocorticoid-inducible leucine zipper (GILZ). These data suggest that the RSV nonstructural proteins mediate RSV repression of GR-induced transactivation and that inhibition of the nonstructural proteins may be a viable target for therapy against RSV-related disease.

Introduction

Respiratory syncytial virus (RSV) is the major cause of severe lower respiratory tract infections in children, resulting in 132,000–172,000 infant hospitalizations/year in the USA (Stockman et al., 2012). Although most children survive in the developed world, there is a significant economic burden associated with RSV disease. Treatment of severe RSV symptoms in children is estimated to cost $600 billion annually in the USA (Paramore et al., 2004). In addition to children, immunocompromised adults and the elderly are also at risk from severe RSV disease (Falsey and Walsh, 2005; Raboni et al., 2003).

RSV is a single-strand negative-sense RNA pneumovirus of the *Paramyxoviridae* family that causes bronchiolitis, an inflammatory disease of the bronchioles. Glucocorticoids, one of the most powerful anti-inflammatory agents available, have no beneficial effect for infants with RSV-induced bronchiolitis (Buckingham et al., 2002; Bulow et al., 1999; Cade et al., 2000; Ermers et al., 2009; Loppow et al., 2001; Panickar et al., 2009; Richter and Seddon, 1998; Roosevelt et al., 1996; Somers et al., 2009). In addition, glucocorticoids show impaired suppression of RSV-induced cytokines *in vitro* (Bonville et al., 2001; Carpenter et al., 2002; Hinzey et al., 2011). These data suggest that RSV may have a deleterious effect on glucocorticoid signaling. In fact, we have recently shown that RSV infection represses glucocorticoid receptor (GR)-mediated gene activation (Hinzey et al., 2011).

Viral infection could interfere with host GR signaling by three potential pathways: production of autocrine factors such as cytokines; activation of other host signaling pathways; or through a direct effect of the viral proteins or RNA. RSV infection of lung epithelial cells results in the production and release of a number of cytokines and in the activation of several intracellular signaling pathways (Garofalo et al., 1996; Lindemans et al., 2006; Mastronarde et al., 1996; Singh et al., 2007; Thomas et al., 2002). In this study we investigated which of these mechanisms RSV utilizes to impair GR function. We show that the RSV nonstructural proteins mediate these repressive actions of RSV infection on GR function through inhibition of mitochrondrial antiviral signaling protein (MAVS).

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Results

Effect of soluble secreted factors on GR-mediated transactivation

RSV induces the expression of soluble cytokines, and cytokines have been shown to play a role in glucocorticoid insensitivity (Hu et al., 2009; Irusen et al., 2002; Ishiguro, 1999; Leung et al., 1995; Matthews et al., 2004; Pace et al., 2011; Sher et al., 1994; Tliba et al., 2008). Therefore, we tested the effect of conditioned media produced from RSV- or mock-infected A549 cells on the ability of dexamethasone to induce a known GR-regulated gene. Analysis of the conditioned media from RSV-infected cells showed increased levels of 27 cytokines, including RANTES, interleukin 6 (IL-6), IL-8 and IL-1α that were not present in conditioned media from mock-infected cells (data not shown). Conditioned media from RSV-infected cells, which also contains live virus, was either applied directly to uninfected cells or UV-irradiated, to inactivate live virus, prior to addition to the uninfected cells. Both of these treatments increased the expression of IL-8 mRNA whereas conditioned media from mock-infected cells did not (Fig. 1A). Dexamethasone induction of GILZ (Fig. 1B) or mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1) (Fig. 1C) was similar in cells treated with conditioned media from mock-infected cells or from RSV-infected cells, either untreated or UV-irradiated.

Role of intracellular signaling pathways in RSV suppression of GR-mediated transactivation

RSV is known to activate a number of intracellular signaling pathways (Garofalo et al., 1996; Lindemans et al., 2006; Mastronarde et al., 1996; Singh et al., 2007; Thomas et al., 2002), therefore, the role of these pathways in RSV-mediated suppression of glucocorticoid induction of a GR-regulated gene was investigated. Specifically, RSV is known to stimulate the Toll-like receptor (TLR) and RIG-like helicase (RLR) pathways (Haeberle et al., 2002; Kurt-Jones et al., 2000; Liu et al., 2007, 2008; Lukacs et al., 2010; Murawski et al., 2009; Segovia et al., 2012). MyD88, the common adaptor molecule of the Toll-like receptors (TLRs), and RIG-I were knocked down by siRNA. RSV infection of A549 cells transfected with siRNAs against MyD88, RIG-I or a control siRNA repressed dexamethasone induction of GILZ to similar levels compared to mock-infected cells (Fig. 2A). Dexamethasone induction of GILZ was slightly higher in mock-infected cells transfected with RIG-I siRNA compared to control siRNA. Knock-down of individual gene expression was confirmed by real-time PCR (Fig. 2B and C).

Knock-down of MAVS, the common adaptor of the RIG-I-like receptors (RLR), reduced dexamethasone induction of GILZ in mock-infected cells compared to control siRNA (Fig. 3A). Glucocorticoid induction of GILZ in cells transfected with MAVS siRNA was further reduced by RSV infection, though this slight effect was not statistically significant. As above, efficiency of siRNA knock-down was confirmed by real-time PCR (Fig. 3B).

Effect of the RSV nonstructural proteins on GR-mediated transactivation

The RSV nonstructural proteins have been shown to inhibit interferon induction by direct interaction with components of the RLR pathway (Boyapalle et al., 2012; Ling et al., 2009), Since siRNA knock-down of MAVS abrogated RSV-mediated inhibition of GR-induced GILZ expression, we tested whether the RSV NS proteins were responsible for the effect of RSV on GR-mediated transactivation. Over-expression of either HA- (Fig. 4A) or FLAG-tagged (Fig. 4B) NS1 repressed dexamethasone induction of the glucocorticoid-responsive promoter MMTVLuc when expressed by transient transfection in Cos7 cells. Over-expression of NS2 or the empty vector had no effect of

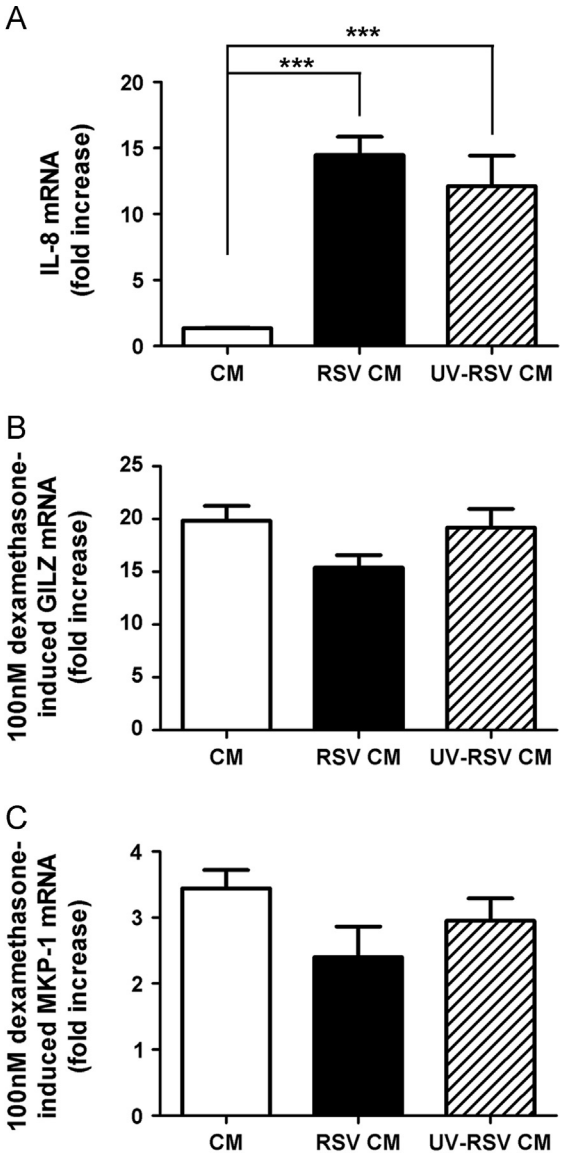

Fig. 1. RSV repression of GR-induced genes is not mediated by a soluble secreted factor. Conditioned media from mock-infected cells (CM) (open bar), RSV-infected cells (RSV CM) (solid bar) or UV-irradiated conditioned media from RSV-infected cells (UV-RSV CM) (hatched bar) was added to uninfected A549 cells. After 5 h vehicle or dexamethasone was added such that the final concentration was 100 nM and cells were incubated for a further 5 h. A. IL-8 mRNA was detected in vehicle only treated cells by real-time PCR, normalized to GAPDH and CAP-1 and compared to untreated cells. Dexamethasone-induced GILZ (B) and MKP-1 (C) mRNA was determined by real-time PCR and normalized to GAPDH and CAP-1. Means and SD are shown ($n=5$). Significance was tested using a 1-way ANOVA and Dunnett post hoc test (***depicts $p < 0.001$).

dexamethasone-induced MMTVLuc activity. Co-expression of NS1 and NS2 repressed dexamethasone-induced MMTVLuc activity similarly to NS1 alone. Expression was confirmed by western blotting using antibodies directed against the HA- or FLAG-tags (Fig. 4C and D). The HA-tagged NS1 was not detected by the HA-tag antibody although there is clearly an effect of transfection of this construct.

To confirm this effect in the context of viral infection, we infected A549 cells with either wild-type recombinant RSV (rA2) or recombinant RSV that lack NS1 (ΔNS1e), NS2 (ΔNS2e), or both NS1 and NS2 (ΔNS1/2e). These recombinant viruses contain GFP inserted in place of the deleted genes. Infection of A549 cells with wild-type rA2 virus repressed dexamethasone-induced GILZ whereas infection with RSV lacking NS1 (ΔNS1e and ΔNS1/2e) had no effect of glucocorticoid-induced GILZ (Fig. 5). Unexpectedly,