Double-stranded RNA causes airway hyperreactivity and neuronal M₂ muscarinic receptor dysfunction

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Bowerfind, William M. L., Allison D. Fryer, and David B. Jacoby. Double-stranded RNA causes airway hyperreactivity and neuronal M₂ muscarinic receptor dysfunction. J Appl Physiol 92: 1417–1422, 2002. First published November 23, 2001; 10.1152/japplphysiol.00934.2001.—Viral infection causes dysfunction of inhibitory M₂ muscarinic receptors (M₂Rs) on parasympathetic nerves, leading to airway hyperreactivity. The mechanisms of M₂R dysfunction are incompletely understood. Double-stranded RNA (dsRNA), a product of viral replication, promotes the expression of interferons. Interferon-γ decreases M₂R gene expression in cultured airway parasympathetic neurons. In this study, guinea pigs were treated with dsRNA (1 mg/kg ip) on 2 consecutive days. Twenty-four hours later, anesthetized guinea pigs had dysfunctional M₂Rs and were hyperresponsive to electrical stimulation of the vagus nerves, in the absence of inflammation. DsRNA did not affect either cholinesterase or the function of postjunctional M₃ muscarinic receptors on smooth muscle. M₂Rs on the nerves supplying the heart were also dysfunctional, but M₂Rs on the heart muscle itself functioned normally. Thus dsRNA causes increased bronchoconstriction and bradycardia via increased release of ACh from the vagus nerves because of loss of M₂R function on parasympathetic nerves in the lungs and heart. Production of dsRNA may be a mechanism by which viruses cause dysfunction of neuronal M₂Rs and airway hyperreactivity.

asthma; interferon; parasympathetic nerves; protein kinase R; viral infection

RESPIRATORY VIRAL INFECTIONS are a major cause of asthma exacerbations. Viruses have been identified in up to 55% of adults (2, 20) and 80–85% of children (17) during asthma attacks. Naturally occurring viral respiratory infections potentiate bronchoconstrictor responses to cold air and histamine in nonasthmatic individuals. Atropine eliminates the potentiation, establishing that the increased airway narrowing is mediated by the parasympathetic nervous system (1, 7). Viral infection potentiates the bronchoconstrictor response to electrical stimulation of the distal, efferent parasympathetic nerves in vagotomized guinea pigs (4), demonstrating that the efferent limb of the reflex arc is hyperfunctional.

In the lungs, parasympathetic nerves provide the dominant control of airway smooth muscle (19). Stimulation of these nerves releases ACh onto postjunctional M₃ muscarinic receptors on airway smooth muscle, causing contraction and bronchoconstriction (22). At the same time, ACh feeds back onto prejunctional inhibitory M₂ muscarinic receptors (M₂Rs) on the nerves, inhibiting further release of ACh and limiting bronchoconstriction (9). Dysfunction of these neuronal M₂Rs increases ACh release and potentiates vagally induced bronchoconstriction. Stimulation of the neuronal M₂Rs with the muscarinic agonist pilocarpine inhibits vagally induced bronchoconstriction by 70–80% by decreasing ACh release (3, 9). Conversely, blocking the M₂R with selective antagonists, such as gallamine, markedly increases bronchoconstriction by increasing ACh release.

Virus infection causes neuronal M₂R dysfunction and airway hyperreactivity (8). The mechanism by which this occurs is only partially understood. Studies in guinea pigs suggest that viruses can inhibit M₂R function via inflammatory cells but also cause loss of M₂R function that is independent of airway inflammation (10).

Rhinovirus, the cause of the common cold, causes primarily upper respiratory tract inflammation and epithelial damage, and yet it is implicated in the majority of virus-associated asthma attacks (2, 16, 20). Although rhinovirus has been shown, by in situ hybridization, to infect bronchial epithelium (21), it replicates principally in the upper airway and has been difficult to culture from the lower airway (5). Furthermore, sputum from allergic subjects with experimental rhinovirus infection has shown either no (12) or only modest increases in inflammatory cells (11).

We suggest that changes in neuronal M₂R expression are mediated by cytokines produced at the sites of infection, which, in the case of rhinovirus, is primarily in the upper airways. These cytokines may then circulate to the airway nerves, where they decrease expression or function of the M₂R and cause airway hyperreactivity. Thus, in the absence of airway inflammation
or local infection, viruses can induce changes resulting in increased bronchoconstriction.

Double-stranded RNA (dsRNA) is produced as a replicative intermediate during viral infection. dsRNA is not normally present in the cytoplasm of mammalian cells. By binding to and activating various host enzymes, it can serve as a potent stimulus to innate host defenses including production of type I interferons (IFNs) (14).

Of primary importance in the host response to viral infection is the transcriptional activation of genes coding for the type I IFNs α and β. These IFNs confer their antiviral effects through the induction of specific IFN-stimulated genes via effects on their common DNA promoter sequence known as the IFNα/β-stimulated response element (23). The response to dsRNA involves release of IFNs, among other cytokines, that limit viral replication (14).

In this study, we tested whether dsRNA causes airway hyperreactivity and M2R dysfunction in vivo, similar to the effects of virus. Direct administration of dsRNA via inhalation or aerosol could cause airway irritation and inflammation and subsequent hyperreactivity. To avoid these complicating effects, dsRNA was given by intraperitoneal injection. This method of exposure establishes a clear anatomic separation between the area of inflammation and the site of neural M2R dysfunction.

METHODS

Animals. Specific pathogen-free female Dunkin-Hartley guinea pigs (300–400 g; supplied by Hilltop Animal Farms, Scottsdale, PA) were shipped in filtered crates and housed in isolation hoods with high-efficiency particulate-filtered air. Guinea pigs were fed a normal diet (Prolab, Agway, Syracuse, NY). Animals were handled in accordance with the standards established by the USA Animal Welfare Acts set forth in National Institute of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

dsRNA exposure. Polyinosinic-polycytidylic acid (Poly[I]-Poly[C]) sodium salt (dsRNA) (Sigma Chemical, St. Louis, MO) was dissolved in sterile water to a concentration of 1 mg/ml. Guinea pigs were injected intraperitoneally with dsRNA (1 mg/kg) on 2 consecutive days. M2R function was tested 1 day after the second injection. Control animals were injected with sterile water (1 ml/kg).

Measurements of pulmonary inflation pressure. Experiments were conducted 1 day after dsRNA treatment. The guinea pigs were anesthetized with urethane (1.7 g/kg ip). This dose produces a deep anesthesia lasting 8–10 h, although none of these experiments lasted longer than 3 h. Both jugular veins were cannulated for the administration of drugs. One endotracheal catheter was cannulated for measurement of blood pressure by using a DTX pressure transducer (Viggo-Spectramed, Oxnard, CA), and the heart rate was derived from the blood pressure tracing by use of a tachograph. The trachea was cannulated, and the animals were ventilated with a positive-pressure, constant-volume rodent respirator (Harvard Apparatus, South Natick, MA) at a tidal volume of 10 ml/kg and a respiratory rate of 100 breaths/min. Animals were paralyzed with succinylcholine (10 μg·kg⁻¹·min⁻¹ iv). All animals were pretreated with guanethidine (10 mg/kg) to deplete norepinephrine. Pulmonary inflation pressure (Ppi) was measured at the trachea via a DTX-pressure transducer. All signals were recorded on a polygraph (Grass Instruments, Quincy, MA). Bronchoconstriction was measured as the increase in Ppi above the baseline inflation pressure produced by the ventilator. The output Ppi signal from the driver of one channel was fed to the preamplifier of a different channel on the polygraph so that increases over baseline could be measured at a higher gain. With this method, increases in Ppi as small as 2–3 mmH₂O can be accurately recorded. Baseline inflation pressure ranged from 90 to 140 mmH₂O.

Studies of vagal responsiveness. Both vagus nerves were cut, and the distal ends were placed on shielded electrodes immersed in mineral oil. Electrical stimulation of both vagi produced bronchoconstriction and bradycardia. The vagus nerves were stimulated at frequencies ranging from 2.0 to 25.0 Hz for 5 s at 120-s intervals, with both pulse duration (0.2 ms) and voltage (10.0 V) kept constant between groups. After the completion of each experiment, atropine (1 mg/kg iv) was administered to confirm that bronchoconstrictions were due to cholinergic nerve stimulation. Changes in Ppi were recorded on a Grass polygraph as described above.

Studies of neuronal M2R function. Baseline responses to electrical stimulation of the vagus nerves were obtained by stimulating both vagus nerves simultaneously at 55-s intervals (2 Hz, 0.2 ms, 7.5–30 V, 44 pulses/train). The function of autoreceptors is frequency dependent. Stimulation of M2Rs by endogenous ACh is greatest at high frequencies of stimulation. Therefore, the ability of exogenous agonists to inhibit vagally induced bronchoconstriction via stimulation of M2Rs is more readily apparent at low frequencies of stimulation (9). Thus the studies with the muscarinic agonist pilocarpine were carried out at 2 Hz. The voltage was chosen at the beginning of each experiment (within a range of 7.5–30.0 V; mean V) to give an increase in pulmonary inflation pressure of ~20 mmH₂O (21.4 ± 0.75 mmH₂O). Cumulative doses of pilocarpine (0.1–100 μg/kg iv) were administered, and the effect on vagally induced bronchoconstriction was measured. Pilocarpine doses of 30–100 μg/kg produced a small, transient bronchoconstriction. Therefore, the effect of these doses of pilocarpine on vagally induced bronchoconstriction was measured after the Ppi had returned to baseline. The results are expressed as a ratio of bronchoconstriction in the presence of pilocarpine to the bronchoconstriction in the absence of pilocarpine. Thus a ratio <1 would indicate that pilocarpine was inhibiting vagally induced bronchoconstriction, demonstrating functional M2Rs. After completion of each experiment, atropine (1 mg/kg iv) was given to confirm that bronchoconstrictions were due to release of ACh onto muscarinic receptors on the smooth muscle.

Studies of postjunctional muscarinic receptor function. The function of muscarinic receptors on airway smooth muscle was tested in vagotomized guinea pigs by measuring bronchoconstriction in response to increasing doses of ACh (1–10 μg/kg iv). A smaller fall in heart rate in response to increasing doses of ACh (1–10 μg/kg iv). A smaller fall in heart rate in response to intravenous ACh in dsRNA-treated animals compared with controls would suggest dysfunction of M2Rs on cardiac myocytes. Binding of ACh to muscarinic M2Rs on cardiac muscle cells causes a fall in heart rate. However, stimulation of M2Rs on the parasympathetic nerves supplying these cardiac muscle cells limits ACh release in a manner identical to the M2Rs on parasympathetic nerves supplying bronchial smooth muscle. The function of these M2Rs was tested in vagotomized guinea pigs by measuring the fall in heart rate in response to increasing doses of ACh (1–10 μg/kg iv). A smaller fall in heart rate in response to intravenous ACh in dsRNA-treated animals compared with controls would suggest dysfunction of M2Rs on cardiac myocytes.
the cardiac muscle. Next, the M$_2$Rs on cardiac muscle cells were tested with increasing frequency of electrical stimulation (2–25 Hz) of the vagus nerves as described above. In this experiment, a greater fall in heart rate, in response to increasing frequency of electrical stimulation of the vagi, in dsRNA-treated animals would suggest dysfunction of the neuronal M$_2$Rs.

**Bronchoalveolar lavage.** At the end of each experiment, bronchoalveolar lavage was performed in situ via the tracheal cannula. The lungs were lavaged with five 10-ml aliquots of PBS. The recovered lavage fluid (35–45 ml) was centrifuged (350 g for 10 min), and the cells were resuspended in 10 ml of PBS. Cells were counted by using a Neubauer hemocytometer (Hauser Scientific, Horsham, PA). Aliquots of the cell suspension were cytospun onto glass slides, stained with Diff-Quik (Baxter Healthcare, McGaw Park, IL), and counted to obtain differential cell counts.

**Drugs and reagents.** ACh, atropine, dsRNA, guanethidine, heparin, pilocarpine, succinylcholine, and urethane were purchased from Sigma Chemical. All drugs were dissolved and diluted in 0.9% NaCl or PBS.

**Statistics.** All data are expressed as means ± SE. ACh, frequency, and pilocarpine responses were analyzed by two-way ANOVA for repeated measures. Baseline heart rates, blood pressures, pulmonary inflation pressures, and changes in pulmonary inflation pressure (before pilocarpine administration), histological measurements, and bronchoalveolar lavage were analyzed by ANOVA (Statview 4.5, Abacus Concepts, Berkeley, CA). A $P$ value of 0.05 was considered significant.

**RESULTS**

**Baseline physiological parameters.** Treatment with dsRNA intraperitoneally on 2 consecutive days did not affect the baseline Ppi, heart rate, or blood pressure.

**Response to vagal stimulation.** Electrical stimulation of the vagus nerves caused frequency-dependent bronchoconstriction (Fig. 1). Control animals had their greatest bronchoconstriction of 274.3 ± 28.2 mmH$_2$O at 25 Hz. Treatment with dsRNA significantly increased vagally induced bronchoconstriction at 25 Hz to 388.0 ± 20.6 mmH$_2$O.

**M$_2$R function.** In control guinea pigs, pilocarpine inhibited vagally induced bronchoconstriction (7.5–30 V, 2 Hz, 0.2 ms, 44 pulses/train) in a dose-dependent manner in controls (○, $n = 8$), demonstrating M$_2$R function. However, pilocarpine did not significantly inhibit vagally induced bronchoconstriction in guinea pigs treated with dsRNA (●, $n = 7$), demonstrating dysfunction of the neuronal inhibitory M$_2$Rs. Values are means ± SE; $P = 0.0005$.

**Bronchoconstriction (Fig. 1).** Control animals had their greatest bronchoconstriction of 274.3 ± 28.2 mmH$_2$O at 25 Hz. Treatment with dsRNA significantly increased vagally induced bronchoconstriction at 25 Hz to 388.0 ± 20.6 mmH$_2$O.

**Acetylcholine (ACh) and succinylcholine (SCH) responses (Fig. 2).** ACh (1–10 mg/kg, i.v.)-induced bronchoconstriction was not different in control (○, $n = 10$) compared with dsRNA-treated guinea pigs (●, $n = 8$). Values are means ± SE.

**Bronchoconstriction (Fig. 3).** Treatment of guinea pigs with dsRNA (1 mg/kg ip) on 2 consecutive days causes airway hyperreactivity in guinea pigs. Simultaneous electrical stimulation of the transected vagus nerves (2–25 Hz, 10 V, 0.2 ms, 5-s train) produces frequency-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure. Frequency-induced bronchoconstriction is significantly increased in guinea pigs treated with dsRNA (●, $n = 11$) compared with controls (○, $n = 9$). Values are means ± SE; $P = 0.0023$. 
with dsRNA the maximal inhibition of vagally induced bronchoconstriction by pilocarpine was 17.6 ± 8.5%, demonstrating that the M₂Rs were dysfunctional.

M₂ receptor function. There was no effect of dsRNA on the response to intravenous ACh, demonstrating that the function of M₃ muscarinic receptors on airway smooth muscle was normal (Fig. 3). This unchanged response to intravenous ACh also demonstrates that smooth muscle contraction was unaffected by dsRNA.

Bronchoalveolar lavage. Treatment of guinea pigs with dsRNA did not change total cells recovered in bronchoalveolar lavage compared with controls. The differential counts were likewise unaffected by dsRNA (Fig. 4).

Heart rate responses. dsRNA-treated guinea pigs had a significantly greater fall in heart rate, compared with control animals, in response to increasing frequency of electrical stimulation of the vagus (Fig. 5A). In contrast, the fall in heart rate with intravenous ACh was not affected by treatment with dsRNA (Fig. 5B).

DISCUSSION

Respiratory virus infection causes airway hyperreactivity and loss of M₂R function (8). In guinea pigs depleted of leukocytes before viral infection, M₂R function is preserved only in animals with low viral titers. Leukocyte-depleted animals with high viral titers had loss of M₂R function (10). This suggests that viruses have effects on M₂Rs that are mediated by leukocytes as well as direct effects that are independent of leukocytes.

We used a synthetic, nonsense double-stranded RNA as a surrogate for virus in an attempt to bypass virus-induced inflammation and to incite the innate host immune system in guinea pigs. dsRNA increased bronchoconstriction in response to electrical stimulation of the vagus nerves (Fig. 1). Potentiation of vagally induced bronchoconstriction was not due to increased M₃ muscarinic receptor activity on airway smooth muscle or to nonspecific increase in smooth muscle contractility because ACh-induced bronchoconstriction was not different between control and dsRNA-treated guinea pigs (Fig. 3). Thus vagally mediated hyperreactivity is due to increased release of ACh. This effect occurred in the absence of airway inflammation (Fig. 4).

In control guinea pigs, pilocarpine inhibited vagally induced bronchoconstriction in a dose-dependent manner (Fig. 2) confirming that the neuronal M₂Rs were functional (9). In contrast, the ability of pilocarpine to inhibit vagally mediated bronchoconstriction was markedly decreased in guinea pigs treated with intraperitoneal dsRNA (Fig. 2). Thus dsRNA inhibits neuronal M₂R function in the lung. M₂R dysfunction caused by dsRNA is independent of airway inflammation (Fig. 4).
There are also M₂Rs both on the cardiac myocytes (postjunctional) and on the parasympathetic nerves supplying the heart (prejunctional) (13). Stimulation of the postjunctional M₂Rs causes a fall in heart rate. The prejunctional M₂Rs function to limit ACh release. Vagally mediated bradycardia was increased by dsRNA (Fig. 5A). In contrast, intravenous ACh-induced bradycardia was not affected by dsRNA (Fig. 5B). This demonstrates that postjunctional M₂Rs on cardiac muscle were not affected by treatment with dsRNA. Thus the accentuated vagally mediated bradycardia in dsRNA-treated animals reflects increased ACh release. This is consistent with loss of M₂R function on the parasympathetic nerves supplying the heart (18).

DsRNA causes M₂R dysfunction on the parasympathetic nerves supplying both the lungs and the heart. In contrast, postjunctional muscarinic receptors, whether M₂ or M₃, were not affected by dsRNA. The finding of normal M₂R function on cardiac muscle in animals with loss of neuronal M₂R function demonstrates that the M₂Rs undergo tissue-specific regulation.

Viral infection causes M₂R dysfunction and airway hyperreactivity (8). Although rhinoviruses cause predominantly upper airway infection, they are implicated more often than any other virus in exacerbation of asthma. Despite very high viral titers in nasal lavage, Calhoun et al. (5), in studies with experimental intranasal rhinovirus infection, were unable to culture virus from the lower airways. Only recently has rhinovirus been clearly shown to infect of bronchial epithelium after nasal inoculation. Using in situ hybridization techniques in bronchial biopsy tissue from subjects exposed to rhinovirus 16, Papadopoulos et al. (21) demonstrated virus localized to the columnar epithelium and basal cell layer. These results were confirmed by RT-PCR for rhinovirus 16. However, lower airway inflammatory responses to rhinovirus infection have generally been absent or modest (11, 12).

Viral infection induces an inflammatory cascade in the host. Clearly, initiation of this response requires recognition that virus is present. dsRNA, a molecule produced by viruses and not normally found in the cytoplasm of mammalian cells, is a powerful stimulus to innate cellular defense mechanisms. RNA viruses (including rhinovirus) produce dsRNA during genomic replication. DNA viruses can also produce dsRNA through the erroneous simultaneous transcription of sense and antisense DNA (14).

DsRNA activates several protein kinases including protein kinase R, a serine/threonine kinase that is induced by type I IFNs (25). These kinases in turn activate a variety of transcription factors, including nuclear factor-κB. Activated nuclear factor-κB translocates into the nucleus and induces the transcription of a number of cellular genes including IFNs, inciting a nonspecific antiviral response (14, 25). Protein kinase R also phosphorylates (thereby inactivating) eukaryotic initiation factor 2α, which results in a generalized inhibition of translation. dsRNA also induces 2’–5’ oligoadenylate synthetases, which in turn activate RNaseL, an RNA-degrading enzyme (25).

IFN-γ decreases expression of M₂Rs in primary parasympathetic nerve cells in culture (15). Although there are distinct effects of the different classes of IFNs on gene expression, there is also a great deal of overlap in the effects caused by the various IFNs (6). Thus the ability of dsRNA to inhibit neuronal M₂R function may be due to decreased M₂R expression by IFNs.

The induction of IFNs through cellular interaction with virally produced dsRNA may be a major mechanism by which rhinoviruses, and other airway viruses, cause M₂R dysfunction and airway hyperreactivity. Intraperitoneal injection of dsRNA is likely endocytozed by inflammatory cells triggering IFN production (24). IFN may then circulate systemically to affect muscarinic receptors on the parasympathetic nerves supplying the airways and the heart.

We have shown that double-stranded RNA injected intraperitoneally into guinea pigs results in M₂R dysfunction and hyperreactivity in the airway in the absence of inflammation. dsRNA also causes dysfunction of the prejunctional muscarinic receptors on parasympathetic nerves supplying the heart. At the same time, the function of the postjunctional muscarinic receptors on the heart is not altered. This shows that M₂Rs are regulated independently in different tissues.

dsRNA mimics the effects of viral infection in causing M₂R dysfunction and airway hyperreactivity. Because dsRNA is produced during viral infection, this may be a fundamental mechanism by which viruses cause M₂R dysfunction. This mechanism may contribute to virus-induced asthma attacks.

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