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Mechanisms of organophosphate insecticide-induced airway hyperreactivity

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In the lung, cholinergic nerves in the vagi mediate airway tone and reactivity. These nerves release acetylcholine onto M3 muscarinic receptors causing contraction of airway smooth muscle, resulting in bronchoconstriction. Vagally induced bronchoconstriction is limited by autoinhibitory M2 muscarinic receptors on parasympathetic nerves (28, 54). Previous studies have shown that neuronal M2 receptors are dysfunctional in animal models of asthma (29, 31, 37) and in patients with asthma (55). Loss of M2 receptor function leads to increased release of acetylcholine from the parasympathetic nerves resulting in potentiation of vagally mediated bronchoconstriction, which contributes to airway hyperreactivity.

Organophosphates are known to alter cholinergic function in the brain (53, 60). The generally accepted mechanism of organophosphate neurotoxicity following acute exposure to high doses is inhibition of acetylcholinesterase (AChE). It has been proposed that this same mechanism underlies the effects of organophosphate insecticides on bronchoconstriction (14, 67). However, there is evidence that in the brain, low-level doses of organophosphate pesticides that do not inhibit AChE may alter cholinergic neurotransmission via direct effects on muscarinic and nicotinic receptor function (1, 7, 36, 39, 40, 42–44). These observations led us to test whether chlorpyrifos (Dursban, Lorsban), a commonly used organophosphate with documented widespread human exposure (15, 17, 25, 34, 48), potentiates vagally induced bronchoconstriction by increasing cholinergic drive in the lungs via inhibition of AChE and/or alteration of neuronal M2 or airway smooth muscle M3 muscarinic receptor function.

METHODS

Animals. Specific pathogen-free male guinea pigs (300–350 g) were shipped from Hilltop Lab Animals (Scottsdale, PA) in filtered crates, housed in high-efficiency, particulate-filtered air, and fed a normal diet (Prolab; Agway, Syracuse, NY). All protocols were approved by The Johns Hopkins University Animal Care and Use Committee.

Chlorpyrifos and eserine exposure. Chlorpyrifos (o,o-diethyl o-[3,5,6-trichloro-2-pyridinol] phosphorothionate, 99.5% pure) was purchased from Chem Service (West Chester, PA) and used within 1 mo of purchase with interim storage as recommended by the manufacturer. Chlorpyrifos dissolved in peanut oil at 70 mg/kg or 390 mg/kg or an equal volume (300 μl) of peanut oil alone was administered to guinea pigs by subcutaneous injection in the subscapular region. Although the most significant routes of chlorpyrifos exposure in humans are oral ingestion and inhalation (30), subcutaneous dosing is commonly used in mechanistic studies of chlorpyrifos and other organophosphates (13, 16, 62, 70). Subcutaneous administration of
chlorpyrifos allows gradual release into the systemic circulation (63), which closely resembles human exposures (30). The guinea pig throat anatomy occludes a stomach tube more so than with rats or mice. Thus the subcutaneous method is also much less stressful to a guinea pig relative to gavage. Animals dosed with chlorpyrifos were monitored for signs of cholinergic intoxication (tremors, altered gait, and excessive excretions) at 1 and 24 h following injections. Guinea pigs given 70 mg/kg sc were tested for airway hyperreactivity 7 days postinjection, whereas animals receiving 390 mg/kg sc were tested 24 h later. These dosing paradigms were found to inhibit lung AChE activity by 0 and 50%, respectively (see Fig. 1), approximating the organophosphate exposures typically observed in humans, which include chronic exposure to low doses that have negligible effect on AChE or acute/subacute exposure to doses that significantly inhibit AChE (30). A 50% inhibition of AChE was chosen as a target value since this level of AChE inhibition is not uncommon in acutely exposed humans (30), and overt toxicity to chlorpyrifos in rodents becomes manifest when brain AChE is inhibited by >60% (57). Vehicle controls treated with peanut oil subcutaneously were tested 7 days later, and since the physiological values obtained with these animals did not differ from those measured in animals that were tested 24 h after saline injections (see Fig. 2), peanut oil controls were not performed for the cohort of animals tested 24 h after chlorpyrifos injection. Eserine was dissolved in sterile saline and administered at a dosage of 0.25 mg/kg iv to anesthetized animals 15 min before physiological measurements, as described below.

Anesthesia and measurement of pulmonary inflation pressure. Guinea pigs were anesthetized (1.5 g/kg urethane ip), and blood pressure and heart rate were measured from the carotid artery. Both jugular veins were cannulated for administration of drugs. Both vagus nerves were cut and placed on electrodes under oil. Succinylcholine (10 μg/kg iv) was infused to paralyze the animals, and they were ventilated via a tracheal cannula with a positive pressure constant volume (1 ml/100 g body wt and 100 breaths/min). Pulmonary inflation pressure (P\textsubscript{pi}) was measured via a side arm at the trachea; bronchoconstriction was measured as the increase in P\textsubscript{pi} over the pressure produced by the ventilator as previously described (28, 29, 37).

Measurement of vagally induced bronchoconstriction. Noradrenaline was depleted 25 min before the start of the experiment with guanethidine (10 mg/kg iv). Both vagi were stimulated (0.2 ms, 10 V, 1–25 Hz, 5-s duration at 2-min intervals) producing frequency-dependent bronchoconstriction and bradycardia due to release of acetylcholine onto postjunctional M2 muscarinic receptors in the heart and postjunctional M3 muscarinic receptors in the lungs. Both vagally induced bronchoconstriction and bradycardia could be abolished by atropine (1 mg/kg iv).

Fig. 2. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2 ms, 5-s train) produced frequency-dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure (P\textsubscript{pi}; □). Vagally induced bronchoconstriction was significantly increased in animals 7 days after a single dose of 70 mg/kg of chlorpyrifos (●) and 24 h after a dose of 390 mg/kg of chlorpyrifos (■). Frequency response curves in animals 7 days after treatment with peanut oil vehicle (○) or acutely with 250 μg/kg of eserine (iv; ●), a nonorganophosphate AChE inhibitor, were not different from control animals. Each point is the mean ± SE of 5–8 animals. *Significantly different from control.

Measurement of neuronal M2 and smooth muscle M3 muscarinic receptor function. The function of neuronal M2 receptors was determined using a standard assay (28, 29, 37): the ability of the muscarinic agonist pilocarpine to inhibit the bronchoconstrictor response to vagal stimulation at 2 Hz. Pilocarpine inhibits vagally induced bronchoconstriction via stimulation of the neuronal M2 receptors at doses that are 100-fold less than the doses required to cause bronchoconstriction by stimulating postjunctional M3 receptors (28). The effect of pilocarpine on vagally induced bronchoconstriction is reported as the ratio of bronchoconstrictor tension in the presence of pilocarpine to bronchoconstriction in the absence of pilocarpine. Decreased inhibition by pilocarpine of vagally induced bronchoconstriction, manifested as a shift to the right of the dose-response curve, indicates M2 receptor dysfunction (28, 29, 37).

In vagotomized guinea pigs, parasympathetic nerves that release acetylcholine and contain M2 receptor are absent. To assess the effects of organophosphates on M3 muscarinic receptor function in airway smooth muscle and to bypass endogenous acetylcholine release from parasympathetic nerves, we measured bronchoconstriction in vagotomized guinea pigs in response to methacholine (1–10 μg/kg iv), which is not readily metabolized by AChE (11, 56), and acetylcholine (1–8 μg/kg iv). The dose-response curve of bronchoconstriction in response to acetylcholine was compared with that of methacholine as well as with vagally induced bronchoconstriction to determine whether changes in vagally induced bronchoconstriction occur via changes in the nerves, in AChE activity, or postjunctional M3 receptor function.

AChE assay. Immediately after the completion of physiological measurements, lungs, brain, and heparinized blood samples were
obtained for determination of AChE activity via the standard Ellman assay (23) using DTNB and acetylthiocholine iodide (ASChI) as the substrate. Assays were run against blanks containing DTNB. The reaction was started with the addition of ASChI after equilibration for 2–3 min. Hydrolysis of ASChI was determined by monitoring the change in absorbance at 405 nm. To inhibit pseudocholinesterase activity, 100 μM tetracisopropyl pyrophosphoramide was included in the assay. Data from lung and brain samples were normalized using protein concentration determined using the bicinchoninic assay according to the manufacturer’s directions (Pierce, Rockford, IL). AChE activity in blood samples was normalized according to the number of red blood cells as determined using a hemacytometer.

**Results.** Data are expressed as means ± SE. Frequency, pilocarpine, methacholine, and acetylcholine dose-response curves were analyzed using a two-way analysis of variance for repeated measures. Baseline heart rates (beats/minute), blood pressures (mmHg), Pp (mmH2O), and changes in Pp (mmH2O before pilocarpine administration) as well as AChE activity levels (as % of control) were significantly inhibited by 50%. Acute exposure to 250 μg/kg of eserine, a nonorganophosphate anticholinesterase, caused a level of AChE inhibition that was not significantly different from that observed with the higher dose of chlorpyrifos (Fig. 1).

Neither chlorpyrifos nor peanut oil altered baseline Pp (control 91 ± 7 mmH2O, peanut oil 96 ± 5 mmH2O, 70 mg/kg of chlorpyrifos 87 ± 7 mmH2O, 390 mg/kg of chlorpyrifos 96 ± 5 mmH2O), resting heart rate (control 281 ± 11 beats/min, peanut oil 271 ± 8 beats/min, 70 mg/kg of chlorpyrifos 268 ± 9 beats/min, 390 mg/kg of chlorpyrifos 300 ± 7 beats/min), or resting diastolic blood pressure (control 45 ± 2.3 mmHg, peanut oil 41 ± 2 mmHg, 70 mg/kg of chlorpyrifos 46 ± 2 mmHg, 390 mg/kg of chlorpyrifos 56 ± 2.4 mmHg) in vagotomized, anesthetized guinea pigs.

Electrical stimulation of both vagi (1–25 Hz) caused a frequency-dependent increase in bronchoconstriction that was significantly potentiated in animals 24 h after a single injection of 390 mg/kg of chlorpyrifos or 7 days after a single injection of 70 mg/kg of chlorpyrifos (Fig. 2). However, a greater increase in bronchoconstriction was observed in animals that received the acute high-dose chlorpyrifos treatment. In contrast, eserine, which significantly inhibited AChE (Fig. 1), did not potentiate vagally induced bronchoconstriction. Vagally induced bronchoconstriction was not altered in animals receiving vehicle alone (peanut oil) relative to control animals.

Neuronal M2 receptor function was tested in chlorpyrifos-treated animals using the muscarinic agonist pilocarpine. Before pilocarpine was administered, simultaneous electrical stimulation of both vagus nerves (2 Hz, 0.2 ms, 5–20 V, 22 s at 1-min intervals) produced transient bronchoconstriction (measured as an increase in Pp) that did not differ among groups (control 27.6 ± 0.2 mmH2O, peanut oil 19.8 ± 3 mmH2O, 70 mg/kg of chlorpyrifos 18.6 ± 5 mmH2O, 390 mg/kg of chlorpyrifos 24.6 ± 6 mmH2O). In guinea pigs treated with peanut oil, pilocarpine (1–100 μg/kg iv) dose dependently inhibited vagally induced bronchoconstriction, demonstrating that the neuronal M2 receptors are functional (Fig. 3, ○). The effect was identical to saline-treated controls (not shown), demonstrating that injection of peanut oil subcutaneously for 7 days did not alter the function of neuronal M2 receptors. The dose-response curve to pilocarpine was shifted significantly to the right in animals 24 h after treatment with chlorpyrifos at 390 mg/kg. A lesser, but still significant, rightward shift was observed 7 days after treatment with 70 mg/kg of chlorpyrifos. Shifting of the pilocarpine dose-response curve to the right is consistent with decreased function of the M2 receptors.

To test the direct response of M3 receptors on airway smooth muscle to muscarinic agonists, bronchoconstriction induced by intravenous methacholine, which is not rapidly metabolized by AChE, and by intravenous acetylcholine was measured in vagotomized guinea pigs. Chlorpyrifos treatment did not alter methacholine-induced bronchoconstriction (Fig. 4A). Because M2 receptors on vagus nerves were not present in these vagotomized guinea pigs, the absence of an effect on methacholine-induced bronchoconstriction indicates that chlorpyrifos does not affect the ability of agonists to interact with postjunctional M3 muscarinic receptors. Acetylcholine-induced bronchoconstriction was significantly increased in animals treated with 390 mg/kg of chlorpyrifos (Fig. 4B). Eserine at a dosage of 250 μg/kg also potentiated acetylcholine-induced bronchoconstriction, and although the eserine dose response did not differ significantly from the control dose-response curve, it also did not differ significantly from the dose-response curve obtained from animals treated with 390 mg/kg of chlorpyrifos. Thus eserine potentiated bronchoconstriction to an intermediate level between that of control chlorpyrifos and that of the control.
animals and animals treated with 390 mg/kg of chlorpyrifos. Treatment of animals with 70 mg/kg of chlorpyrifos for 7 days had no effect on acetylcholine-induced bronchoconstriction. The potentiation of acetylcholine-induced bronchoconstriction by the higher dose of chlorpyrifos and by eserine is consistent with the inhibition of AChE by these treatments (see Fig. 1).

M2 muscarinic receptors are also present in the heart. Thus we measured vagally induced bradycardia to determine whether organophosphate insecticides alter M2 muscarinic receptors in tissues other than the lung. In the heart, stimulation of the vagus nerves (1–25 Hz) produces bradycardia that is frequency dependent (Fig. 5). Treatment with 70 mg/kg of chlorpyrifos did not alter the vagally induced fall in heart rate relative to control. However, in the animals treated with 390 mg/kg of chlorpyrifos for 24 h, the frequency response curve was potentiated at frequencies <20 Hz (Fig. 5). At 20 and 25 Hz, the fall in heart rate approaches maximum, and the differences are no longer significant. However, the potentiation of vagally induced bradycardia by the higher dose of chlorpyrifos does not appear to be mediated by inhibition of AChE since eserine at a concentration that significantly inhibits AChE does not potentiate vagally induced bradycardia (Fig. 5). Methacholine- and acetylcholine-induced bradycardia was not altered by either high- or low-dose chlorpyrifos treatment (Fig. 6) despite inhibition of AChE by the higher dose of chlorpyrifos.

DISCUSSION

In humans, exposure to organophosphate insecticides and other pesticides has been associated with a variety of respiratory symptoms, including decreased forced expiratory volume in 1 min, wheeze, cough, and shortness of breath (3, 19, 35, 46, 47, 65, 67). Similarly, it has been reported that organophosphate insecticides induce bronchospasm in a variety of animals (20, 32, 66). Our data provide more direct evidence of a causal link between organophosphate exposure and airway hyperreactivity. Specifically, we observed that vagally induced bronchoconstriction in guinea pig lungs is significantly potentiated...
following either acute (24 h after 390 mg/kg of chlorpyrifos) or chronic (7 days after 70 mg/kg) exposure to chlorpyrifos.

It has been proposed that if organophosphates contribute to asthma, it is likely due to inhibition of AChE (14, 24, 67), which is the principal mechanism underlying acute organophosphate neurotoxicity. Support for this hypothesis includes observations that not only organophosphate insecticides, but also other structurally unrelated AChE-inhibiting insecticides, such as carbaryl, enhance airway hyperreactivity in rats (20) and humans (67). However, two observations from our studies suggest mechanisms other than AChE inhibition mediate chlorpyrifos effects on vagally induced bronchoconstriction. First, our data indicate that animals tested 7 days after receiving 70 mg/kg of chlorpyrifos (subcutaneously) exhibit airway hyperreactivity in the absence of AChE inhibition. Second, acute administration of the nonorganophosphate eserine at a dose (250 μg/ml) that significantly inhibits AChE does not potentiate vagally induced bronchoconstriction. Similarly, a poor correlation has been noted between cholinesterase inhibition and toxic effects in the brain by some organophosphate insecticides (22), prompting investigations of alternative mechanisms of neurotoxicity. These observations are important because they suggest that toxicity from some organophosphates may occur below thresholds of exposure normally defined by AChE inhibition.

Neuronal M2 muscarinic receptors limit release of acetylcholine from the vagus nerves in the lungs (28). Pharmacological blockade of neuronal M2 receptors increases release of acetylcholine from the nerves (5, 27), which potentiates vagally induced bronchoconstriction (28, 29, 37). Our data show that neuronal M2 receptor function is inhibited by both high and low doses of chlorpyrifos, consistent with other findings that organophosphate insecticides act on muscarinic receptors in the brain (1, 36, 38, 43). This effect of chlorpyrifos on vagally induced bronchoconstriction is dependent on the dosing regimen. Vagally induced bronchoconstriction was significantly greater in animals treated with the high dose of chlorpyrifos relative to animals treated with the low dose (Fig. 2). A similar dependency was observed for the effects of chlorpyrifos on M2 receptor function as determined by pilocarpine dose-response curves (Fig. 3). In contrast, neither dose of chlorpyrifos changed the response to intravenous methacholine, demonstrating that the function of M3 muscarinic receptors on airway smooth muscle was not altered in animals in which M2 receptors mediating ACh release were not present. Selective loss of neuronal M2 receptor function in the lungs is also associated with other models of airway hyperreactivity, including antigen challenge (29), viral infection (37), and exposure to ozone (31), suggesting that decreased M2 receptor function on airway nerves is a generalized mechanism underlying airway hyperreactivity.

The ability of organophosphate insecticides to inhibit neuronal M2 receptors may not be restricted to the lungs. Organophosphate insecticides have been shown to inhibit muscarinic receptor binding in the brain (1, 7, 36, 39, 40, 43, 44) and bind to a subpopulation of M2 receptors in the heart (68). In the heart, M2 receptors are present on parasympathetic nerves that supply the heart where they function to inhibit release of acetylcholine (52, 58) as well as on cardiac muscle where they mediate bradycardia (10, 51). The single high dose of chlorpyrifos potentiated vagally induced bradycardia but not bradycardia induced by intravenous administration of acetylcholine. This is consistent with loss of neuronal M2 receptor function on parasympathetic nerves in the heart resulting in increased release of acetylcholine, which potentiates vagally induced bradycardia. Neither dose of chlorpyrifos altered bradycardia induced by acetylcholine or methacholine administered intravenously, indicating that the postjunctural M2 receptors are not sensitive to organophosphate insecticides, and this is consistent with the sensitivity of neuronal M2 receptors we observed in the lung. These data are also consistent with previous observations that the function of the neuronal M2 receptors in the heart are inhibited by systemic administration of double-stranded RNA, which does not alter the function of postjunctural M2 receptors (9). Thus the neuronal receptors appear to be more vulnerable to inhibition than the postjunctural receptors.

The mechanism(s) by which organophosphate insecticides alter M2 receptor function in the lungs have yet to be elucidated. Mechanisms by which these compounds alter muscarinic receptor function in neurons include downregulation of muscarinic receptor expression (39, 40), modulation of ligand binding to muscarinic receptors (38, 43, 44), and alteration of signal transduction pathways downstream of muscarinic receptor activation (8, 36, 72). In vitro studies of cardiac M2 muscarinic receptors have demonstrated that acute exposure to the oxon metabolite of chlorpyrifos alters ligand binding via diethylphosphorylation of the receptor itself (7). Whether these mechanisms underlie the effects of organophosphates on neuronal M2 receptor function in the lung has yet to be determined.

Data presented here indicate that organophosphate insecticides potentiate vagally induced bronchoconstriction via disruption of the cholinergic control of airway responsiveness. A significant finding from our studies is that chlorpyrifos altered neuronal M2 receptor function in the lung at concentrations that did not inhibit AChE. Although the threshold concentration for this effect was not determined in our studies, it has been shown that ligand binding to muscarinic receptors in the brain (43) as well as signaling pathways downstream of muscarinic receptor binding (64) can be disrupted by very low (nanomolar to picomolar) concentrations of organophosphate insecticides. These data suggest that exposure to not only occupational, but also environmental, levels of these compounds may have biological consequences. The significance of these findings to public health is heightened by evidence of widespread human exposure to chlorpyrifos and other organophosphate insecticides (34, 45).

Children represent a potentially sensitive subpopulation with respect to asthma. Thus it is of great concern that in a sample of 84,000 children across the United States, the urinary levels of chlorpyrifos metabolites were above the detection limit 98% of the time, compared with a 4% detection rate for the herbicide atrazine (2). Many of the organophosphate insecticides have been restricted or banned due to their developmental neurotoxicity in animals. However, many of these compounds, including chlorpyrifos, are still used commercially in both agricultural settings and urban environments. These pesticide usage patterns correlate positively with reports of high incidence of asthma morbidity in agricultural workers (3, 35, 41, 46, 67, 69, 76) and in residents of the inner cities of the United States (18, 33, 49, 61). The coincident rise in the incidence of
asthma (33) and in the use of organophosphate insecticides (26, 45, 71, 75) suggests a potential causal relationship between these two observations, which is corroborated by our data obtained using an animal model of airway hyperreactivity. Our data raise significant questions regarding the current use of organophosphate insecticides in the inner cities to control cockroach antigen (6, 15, 50, 73, 74), which itself has been associated with asthma (4, 21), and suggest that exposure to insecticides may be contributing to rather than ameliorating asthma.

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