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Research report

Neonatal parathion exposure and interactions with a high-fat diet in adulthood: Adenylyl cyclase-mediated cell signaling in heart, liver and cerebellum

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ABSTRACT

Organophosphates are developmental neurotoxicants but recent evidence points to additional adverse effects on metabolism and cardiovascular function. One common mechanism is disrupted cell signaling mediated through cyclic AMP, targeting neurohumoral receptors, G-proteins and adenylyl cyclase (AC) itself. Earlier, we showed that neonatal parathion evokes later upregulation of the hepatic AC pathway in adolescence but that the effect wanes by young adulthood; nevertheless metabolic changes resembling prediabetes persist. Here, we administered parathion to neonatal rats (postnatal days 1–4, 0.1 or 0.2 mg/kg/day), straddling the threshold for cholinesterase inhibition, but we extended the studies to much later, 5 months of age. In addition, we investigated whether metabolic challenge imposed by consuming a high-fat diet for 7 weeks would exacerbate neonatal parathion's effects. Parathion alone increased the expression or function of G_i, thus reducing AC responses to fluoride. Receptors controlling AC activity were also affected: β-adrenergic receptors (βARs) in skeletal muscle were increased, whereas those in the heart were decreased, and the latter also showed an elevation of m_2-muscarinic acetylcholine receptors, which inhibit AC. The high-fat diet also induced changes in AC signaling, enhancing the hepatic AC response to glucagon while impairing the cardiac response to fluoride or forskolin, and suppressing βARs and m_2-muscarinic receptors; the only change in the cerebellum was a decrease in βARs. Although there were no significant interactions between neonatal parathion exposure and a high-fat diet, their convergent effects on the same signaling cascade indicate that early OP exposure, separately or combination with dietary factors, may contribute to the worldwide increase in the incidence of obesity and diabetes.

1. Introduction

Organophosphate (OP) pesticides represent nearly half of worldwide insecticide use [4] but are undergoing increased scrutiny because of their propensity to elicit developmental neurotoxicity at levels that are below the threshold for acute signs of exposure, and even doses lower than those required for cholinesterase inhibition, the mechanism that is used for biomonitoring [8,22,23,27]. Pregnant women and young children are typically exposed to OPs under these low-dose conditions [5,9,17] and recent findings confirm that such exposures can produce long-term neurobehavioral impairment [6,7,19,21]. Although the systemic toxicity and signs of OP intoxication reflect their shared ability to inhibit cholinesterase [16,18], other important mechanisms of toxicity clearly exist. Recent data point to dysregulation of cell signaling cascades as one of the critical targets that contribute to the adverse outcomes seen at lower exposures [8,22,23,27]. Chief among these is the pathway that generates cyclic AMP, a critical second messenger that mediates numerous neurotransmitter and hormonal receptor signals; these are linked to the generation of cyclic AMP through G-proteins that regulate adenylyl cyclase (AC), the enzyme that synthesizes cyclic AMP from ATP.

The impact of OPs on AC signaling is critically important for effects outside the central nervous system, since cyclic AMP controls cell function in all the organs and tissues involved in metabolic and cardiovascular homeostasis. In adult rats, chlorpyrifos exposures exceeding the threshold for cholinesterase inhibition lead to enhanced weight gain [14] and diabetes-like changes in hepatic energy metabolism [1]. At lower doses administered during development, chlorpyrifos also produces excess weight gain and dysregulation of leptin [11], along with a metabolic profile resembling prediabetes [24]. Similarly, when we exposed neonatal rats to parathion at doses straddling the threshold for barely detectable cholinesterase inhibition, we found later emer-

Abbreviations: AC, adenylyl cyclase; ANOVA, analysis of variance; βAR, β-adrenergic receptor; m2AChR, m2-muscarinic acetylcholine receptor; OP, organophosphate.

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gence of a prediabetes-like state, involving excessive weight gain, hyperglycemia, abnormalities of lipid metabolism and adipose tissue inflammation [12,13]. Further, many of the metabolic effects of early-life OP exposure were exacerbated when animals were switched to a high-fat diet in adulthood, including a much greater fat-induced weight gain than that seen with the equivalent dietary change in controls [12,13,20].

Although OPs are likely to disrupt metabolism and cardiovascular function at many different levels, one common feature is their ability to produce lasting changes in AC signaling, involving a net gain of pathway function, an effect noted for chlorpyrifos, diazinon and parathion [2,15]. For all three, early-life OP exposure produced heterologous sensitization of the AC pathway later in life, with parallel increases in all aspects of activity, ranging from receptor-mediated stimulation, through effects on G-protein activity, and on the expression or catalytic activity of AC itself. However, there was a difference in that the effects of parathion on AC signaling waned between adolescence and young adulthood, whereas those of diazinon and chlorpyrifos did not [2,15]. Nevertheless, the metabolic consequences of neonatal parathion exposure persisted [12,13], suggesting either that the earlier changes are sufficient to reprogram metabolic function, or alternatively that the AC changes are not critical to parathion's effects on metabolism. The latter seems highly unlikely, given that cyclic AMP is one of the primary mediators of hepatic gluconeogenesis, glycogenolysis and lipolysis. In the current study, we explored whether the additional metabolic stress imposed by consuming a high-fat diet in adulthood could unmask persistent effects of neonatal parathion exposure on AC signaling, an approach modeled after our earlier studies on metabolic effects of OPs [12,13,20]. As before, we focused on parathion treatment regimens straddling the threshold for cholinesterase inhibition [26] and then in adulthood, we switched half the animals to a high-fat diet that more than doubles serum β-hydroxybutyrate concentrations [13]. To assess the impact on AC signaling, we evaluated function at each step in the cascade: basal AC activity, the responses mediated by β-adrenergic receptors (βAR) and glucagon receptors, the response to G-protein activation by fluoride, and activity with maximal activation of AC itself by forskolin [10]. Further, we measured ligand binding for βARs and for the inhibitory m_2-muscarinic acetylcholine receptors (m_2AChRs). We conducted our studies in peripheral tissues (heart, liver) and compared the effects to those seen in a brain region (cerebellum) that shows a similar AC response to βAR activation. Finally, we also assessed βAR binding in skeletal muscle (gastrocnemius) to determine whether this prominent site of energy utilization was affected by parathion or the high-fat diet, separately or together.

2. Materials and methods

2.1. Animal treatments and diet

All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the Duke University Institutional Animal Care and Use Committee and in accordance with all federal and state guidelines. Timed-pregnant Sprague–Dawley rats were housed in breeding cages, with a 12 h light–dark cycle and free access to water and food (PMI LabDiet 5001). On the day after birth, all pups were randomized and redistributed to the dams with a litter size of 10 (5 males, 5 females) to maintain a standard nutritional status. Parathion was dissolved in dimethylsulfoxide to provide consistent absorption [26,28,30] and was injected subcutaneously in a volume of 1 ml/kg once daily on postnatal days 1–4; control animals received equivalent injections of the dimethylsulfoxide vehicle. Doses of 0.1 and 0.2 mg/kg/day were chosen because they straddle the threshold for barely detectable cholinesterase inhibition and the first signs of reduced weight gain or impaired viability [26,28]. To avoid the possibility that dams might distinguish between control and parathion-treated pups, all pups in a given litter received the same treatment. Randomization of pup litter assignments within treatment groups was repeated at intervals of several days up until weaning, and in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Offspring were weaned on postnatal day 21 and the final litter assignment for each rat was noted. Studies were conducted using one male and one female from each final litter, with 6 animals for each group, defining a group as a specific neonatal treatment, dietary condition and sex. After weaning, animals were separated by sex and housed in groups according to standard guidelines.

Beginning at 15 weeks of age, half the rats were switched to a high-fat diet (OpenSource D12330), providing 58% of total calories as fat; 93% of the fat is hydrogenated coconut oil. The remaining rats continued on the standard LabDiet 5001 diet, which provides 13.5% of total calories as fat; with this diet, 27% of the fat is saturated. Although the high-fat diet contains 37% more calories per gram, we found that animals on this diet reduce their food intake by approximately the same proportion [13], so that the total dietary intake is isocaloric; nevertheless, animals gain excess weight when fed a diet with a higher fat content [13]. During the 24th postnatal week, animals from each of the finally assigned litters were decapitated and the heart, one liver lobe (the same lobe from each animal), cerebellum and gastrocnemius muscle were dissected, blotted, frozen in liquid nitrogen and maintained at −45 °C.

2.2. Assays

Tissues were thawed and homogenized (Polytron; Brinkmann Instruments, Westbury, NY) in buffer containing 145 mM sodium chloride, 2 mM magnesium chloride, and 20 mM Tris (pH 7.5), strained through several layers of cheesecloth to remove connective tissue, and the homogenates were then sedimented at 40,000 × *g* for 15 min. The pellets were washed twice and then resuspended in 250 mM sucrose, 2 mM $MgCl_2$, and 50 mM Tris. For determinations of AC activity, aliquots of the membrane preparation were incubated for 30 min (heart, liver) or 10 min (cerebellum) at 30 °C with final concentrations of 100 mM Tris–HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM $MgCl_2$, 10 μM GTP, 1 mg/ml bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/ml phosphocreatine kinase. The enzymatic reaction was stopped by heating and sedimentation, and the supernatant solution was then assayed for cyclic AMP using commercial radioimmunoassay or immunoassay kits; the two types of kits gave equivalent results. In addition to assessing basal AC activity, we evaluated responses to 100 μM isoproterenol, 3 μM glucagon, 10 mM NaF and 100 μM forskolin. These concentrations produce maximal responses to each stimulant as assessed in earlier studies [3,31,32].

To evaluate βAR binding, aliquots of the same membrane preparation were incubated with 67 pM [^{125}I]-iodopindolol in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM sodium ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature; samples were evaluated with and without 100 μM isoproterenol to displace specific binding. Incubations were stopped by addition of 3 ml ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters, which were washed with additional buffer and counted by liquid scintillation spectrometry. For cardiac m_2AChR binding, the membrane suspension was reconstituted in 10 mM sodium-potassium phosphate buffer (pH 7.4) and incubated with 1 nM [^{3}H] AFDX384, with or without 1 μM atropine to displace specific binding.

We did not assess m_2AChR binding in the liver, cerebellum or gastrocnemius muscle because of the sparsity of the receptors in these tissues. We conducted preliminary studies of AC activity in the gastrocnemius muscle and found extremely variable results; accordingly, these determinations also were not carried out in the present work. Similarly, we did not evaluate the effects of glucagon in the cerebellum, a tissue in which this metabolic hormone has no defined biologic role.

2.3. Data analysis

Data were compiled as means and standard errors. The experimental design was identical to that in two previous papers using animals from the same treatment cohorts [13,25], so the overall statistical procedures were the same and will be described only briefly here. Each set of determinations began with a global ANOVA incorporating all variables in a single test: neonatal treatment, diet, sex, tissue and the multiple dependent measures made for each class of variables, with the latter regarded as repeated measures (since multiple measurements were made from the same tissue sample). Data were log-transformed because of heterogeneous variance contributed by tissue, diet and sex. When this initial test showed main effects of the contributing variables as well as significant interactions among the variables, we then conducted a series of nested, lower-order ANOVAs separating the measurements according to the interactive variables. Where permitted, the lower-order tests were followed by Fisher's protected least significant difference to establish individual groups that differed from the corresponding control. For all tests, significance was assumed at $p < 0.05$. However, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables [29]. The criterion for interaction terms was not used to assign significance to the effects but rather to identify interactive variables requiring subdivision for lower-order tests of main effects of parathion or the high-fat diet, the variables of chief interest. Where treatment effects were not interactive with other variables, we report only the main treatment effects without performing lower-order analyses of individual values.

To ensure that treatment and diet effects could be compared across all groups, all assays were conducted simultaneously on all samples for a given tissue and sex, but technical limitations dictated that each tissue and sex had to be performed in divided