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Neonatal dexamethasone treatment leads to alterations in cell signaling cascades controlling hepatic and cardiac function in adulthood

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ABSTRACT

Increasing evidence indicates that early-life glucocorticoid exposure, either involving stress or the therapy of preterm labor, contributes to metabolic and cardiovascular disorders in adulthood. We investigated cellular mechanisms underlying these effects by administering dexamethasone (DEX) to neonatal rats on postnatal (PN) days 1–3 or 7–9, using doses spanning the threshold for somatic growth impairment: 0.05, 0.2 and 0.8 mg/kg. In adulthood, we assessed the effects on hepatic and cardiac cell function mediated through the adenylyl cyclase (AC) signaling cascade, which controls neuronal and hormonal inputs that regulate hepatic glucose metabolism and cardiac contractility. Treatment on PN1–3 produced heterologous sensitization of hepatic signaling, with upregulation of AC itself leading to parallel increases in the responses to β-adrenergic or glucagon receptor stimulation, or to activation of G-proteins by fluoride. The effects were seen at the lowest dose but increasing DEX past the point of somatic growth impairment led to loss of the effect in females. Nonmonotonic effects were also present in the heart, where males showed AC sensitization at the lowest dose, with decreasing effects as the dose was raised; females showed progressive deficits of cardiac AC activity. Shifting the exposure to PN7–9 still elicited AC sensitization but with a greater offsetting contribution at the higher doses. Our findings show that, in contrast to growth restriction, the glucocorticoids associated with stress or the therapy of preterm labor are more sensitive and more important contributors to the cellular abnormalities underlying subsequent metabolic and cardiovascular dysfunction.

1. Introduction

Adverse events during fetal development contribute to subsequent metabolic and cardiovascular disease in adulthood. Barker linked low birth weight per se to disease outcomes [3], and excess glucocorticoid exposure associated with prenatal stress is thought to play a mechanistic role in this connection [4,9,31]. The societal impact of prenatal glucocorticoid exposure is increasingly important because of the expanded use of these agents in preterm labor [13], a treatment currently involving 10% of all US pregnancies [26]. Although glucocorticoids enhance lung maturation and thus prevent neonatal respiratory distress syndrome, such treatments are now implicated in subsequent hypertension, hyperglycemia, hyperinsulinemia, altered behavior and neuroendocrine responses, all of which can emerge throughout the lifespan after periods of apparent normality [32,34].

Notwithstanding these epidemiological relationships, few studies have provided a mechanistic understanding of how early glucocorticoid exposure leads to metabolic or cardiovascular dysfunction. Although there are numerous reports of glucocorticoid effects in animal models, most of these involve relatively high doses that elicit persistent stunting of somatic growth, outright cerebral atrophy and endocrine disruption [6,10–12,14,24–28,41,42], as well as causing a loss of insulin response similar to that in diabetes [8,15,32,33,43]. This still leaves major uncertainties as to glucocorticoid effects relevant to stress, or at or below the threshold for typical therapeutic use in preterm infants. In a series of recent studies, we showed that, even at subtherapeutic doses, dexamethasone (DEX) exposure in fetal and neonatal rats compromises key aspects of brain development when given during specific critical periods corresponding to the selfsame developmental stages recommended for preterm infants [18–20,39]. Here, we have used the same approach to address cell signaling in peripheral tissues that are the likely targets for the emergence of metabolic and cardiovascular disorders, the liver and heart.

We focused on the key role played by the adenylyl cyclase (AC) cascade, which governs the formation of cyclic AMP, the second messenger that controls hepatic gluconeogenesis and glycogenolysis, that modulates insulin function, and that regulates heart rate and contractility. In the liver, β-adrenergic receptors (βARs) and glucagon receptors act through the stimulatory G-protein, G_s, to activate AC, thus eliciting gluconeogenesis and lipolysis. The importance of this pathway was recently emphasized by studies showing that neonatal

Abbreviations: AC, adenylyl cyclase; ANOVA, analysis of variance; βAR, β-adrenergic receptor; DEX, Dexamethasone; m_2AChR, m_2-muscarinic acetylcholine receptor; PN, postnatal day.

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exposures to organophosphate pesticides produce sensitization of hepatic AC to these receptor inputs, leading ultimately to metabolic abnormalities resembling those in prediabetes [22,29,38,39]. Similarly, prediabetic abnormalities and altered susceptibility to development of diabetes have been identified in humans with AC gene polymorphisms [30]. Accordingly, we evaluated the effects of DEX exposure of neonatal rats in two treatment periods, postnatal days (PN) 1–3 and 7–9, bracketing stages of development in the rat that are equivalent to those in second- to early-third trimester human fetuses, the stage in which glucocorticoid use is recommended for preterm infants [13]; we focused on doses within the therapeutic range (0.2 or 0.8 mg/kg) as well as a much lower dose (0.05 mg/kg) likely to be more representative of stress-related glucocorticoid actions. The three-day regimens were chosen to correspond to multiple glucocorticoid courses, as used in approximately 85% of all cases of preterm delivery [7]. We then evaluated the impact on the AC signaling cascade in adulthood (PN75), focusing on each individual step in the pathway (Fig. 1); the studies were modeled after our earlier work on organophosphates [2,29]. In addition to assessing the effects on basal AC activity, we evaluated the response to βARs and glucagon receptors, both of which stimulate AC through via activation of the stimulatory G-protein, G_s. We also determined the effect of fluoride, which evokes maximal activation of both G_s and the corresponding inhibitory protein, G_i. We then measured the maximal activation of AC itself by forskolin, which acts directly on the enzyme by binding to the catalytic core [17]. Finally, we measured ligand binding for βARs and for the inhibitory m_2-muscarinic acetylcholine receptors (m_2AChRs).

2. Materials and methods

2.1. Animal treatments

All experiments were carried out humanely and with due regard for alleviation of suffering, with protocols approved by the 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 food and water. On the day of birth, all pups were randomized and redistributed to the dams with a litter size of 10 to maintain a standard nutritional status. Pups received daily subcutaneous injections of DEX phosphate (0.05, 0.2, or 0.8 mg/kg) on PN1–3 or PN7–9, whereas controls received equivalent volumes (1 ml/kg) of isotonic saline vehicle. On each day of treatment and at intervals of several days thereafter, pups were re-randomized within their respective treatment groups and in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Offspring were weaned on PN21. On PN75, one male and one female from each of the finally-assigned litters were decapitated and the heart and one liver lobe were dissected, blotted, frozen in liquid nitrogen and maintained at −45 °C.

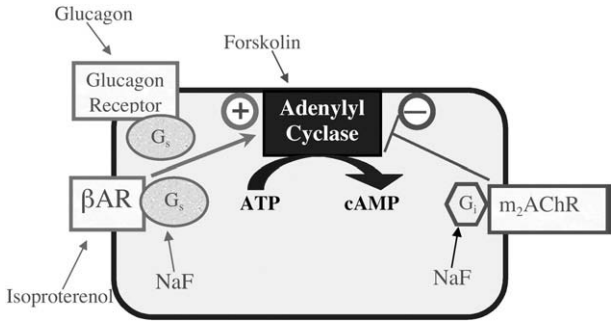

Fig. 1. Mechanisms controlling AC activity, showing probes for each step in the pathway: isoproterenol for the βAR, glucagon for the glucagon receptor, NaF for the G-proteins, and forskolin for AC itself. Both βARs and glucagon receptors enhance AC activity through the stimulatory G-protein, G_s, whereas m_2AChRs inhibit AC through mediation of the inhibitory protein, G_i.

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 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 [2,44,45].

For the ligand binding determinations, there were technical limitations imposed by the large number of membrane preparations that had to be examined. The overall strategy was to determine binding at a single, subsaturating ligand concentration to enable the detection of changes that originate either in altered K_d or B_{max}. 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; determinations were not done in the liver, since this tissue is sparse in m_2AChRs and lacks sufficient AC response to m_2AChR agonists.

2.3. Data analysis

Data are presented as means and standard errors obtained from 6 animals in each treatment group for each sex and treatment regimen. To establish treatment differences, a global analysis of variance (ANOVA; data log transformed because of heterogeneous variance across tissues and AC stimulants) was first conducted for all variables: the *in vivo* treatment groups (control vs. DEX doses), sex, tissue and the stimulant condition under which the measurement was made (basal AC, isoproterenol-stimulated AC, glucagon-stimulated AC, fluoride-stimulated AC, forskolin-stimulated AC); the latter was considered to be a repeated measure because the same membrane preparation was used for each of the multiple assay conditions. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC stimulant responses that differed from control values; these were conducted by lower-order ANOVAs, followed, where appropriate, by Fisher's Protected Least Significant Difference Test to identify individual values for which the DEX groups differed from the corresponding control. For all tests, significance for main treatment effects was assumed at $p < 0.05$. However, for interactions at