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Long-term consequences of disrupting adenosine signaling during embryonic development

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ABSTRACT

There is growing evidence that disruption in the prenatal environment can have long-lasting effects on an individual's health in adulthood. Research on the fetal programming of adult diseases, including cardiovascular disease, focuses on epi-mutations, which alter the normal pattern of epigenetic factors such as DNA methylation, miRNA expression, or chromatin modification, rather than traditional genetic alteration. Thus, understanding how *in utero* chemical exposures alter epigenetics and lead to adult disease is of considerable public health concern.

Few signaling molecules have the potential to influence the developing mammal as the nucleoside adenosine. Adenosine levels increase rapidly with tissue hypoxia and inflammation. Adenosine antagonists including the methlyxanthines caffeine and theophylline are widely consumed during pregnancy. The receptors that transduce adenosine action are the A1, A2a, A2b, and A3 adenosine receptors (ARs). We examined the long-term effects of *in utero* disruption of adenosine signaling on cardiac gene expression, morphology, and function in adult offspring.

One substance that fetuses are frequently exposed to is caffeine, which is a non-selective adenosine receptor antagonist. Over the past several years, we examined the role of adenosine signaling during embryogenesis and cardiac development. We discovered that *in utero* alteration in adenosine action leads to adverse effects on embryonic and adult murine hearts. We find that cardiac A1ARs protect the embryo from *in utero* hypoxic stress, a condition that causes an increase in adenosine levels. After birth in mice, we observed that *in utero* caffeine exposure leads to abnormal cardiac function and morphology in adults, including an impaired response to β-adrenergic stimulation. Recently, we observed that *in utero* caffeine exposure induces transgenerational effects on cardiac morphology, function, and gene expression.

Our findings indicate that the effects of altered adenosine signaling are dependent on signaling through the A1ARs and timing of disruption. In addition, the long-term effects of altered adenosine signaling appear to be mediated by alterations in DNA methylation, an epigenetic process critical for normal development.

1. Fetal programming of adult disease

It is well recognized that disruption of the intrauterine environment by nutritional or chemical factors may influence the fetus, resulting in long-term adverse effects after birth and into adulthood (Cetin et al., 2013; Gluckman et al., 2005; Vo and Hardy, 2012; Barker, 2008). By disrupting normal prenatal development, environmental factors and chemical exposures lead to the fetal programming of adult disease, including cardiovascular disease (Barker, 1990, 1999; Gluckman et al., 2009). The timing of the *in utero* insult is important and can affect the outcomes in adulthood (Gluckman et al., 2009).

Fetal programming of adult disease involves several potential mechanisms, including genetic and non-genetic events. Non-genetic factors include influences on cell division (Porrello et al., 2008), while genetic factors include epigenetic influences on gene activity and expression (Simmons, 2005, 2007a; Jirtle and Skinner, 2007; Callinan and Feinberg, 2006; Bjornsson et al., 2004). Epigenetic changes not only affect the exposed embryo, but may also

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affect subsequent generations and contribute to the development of disease, as shown by endocrine disruptors vinclozolin and bisphenol A Manikkam et al., 2012a, Guerrero-Bosagna et al., 2012. Research in our laboratory has demonstrated that *in utero* caffeine exposure disrupts adenosine action and leads to long-term adverse effects on cardiac function (Buscariollo et al., 2014; Wendler et al., 2009; Fang et al., 2016). Furthermore, depending on the timing of exposure, caffeine has transgenerational effects on cardiac function (Buscariollo et al., 2014).

2. Adenosine

Adenosine consists of an adenine group attached to a ribose moiety. Adenosine is present in all cells and is a component of nucleic acids and energy-carrying molecules (Jacobson, 2009; Rivkees et al., 2001). Adenosine can be directly released from the cell or generated extracellularly (Eckle et al., 2009).

It is likely that several different humoral agents can transduce environmental effects on the embryo and developing fetus. However, adenosine is particularly attractive to study for several reasons. First, adenosine levels are dynamically regulated and increase markedly with tissue hypoxia and energy depletion (Ijzerman et al., 1997). Adenosine receptors may play an important role in sensing disruptions in the intrauterine environment, as it is present in all cells and is a component of nucleic acids and energy-carrying molecules (Jacobson, 2009; Rivkees et al., 2001). For example, under basal conditions the interstitial adenosine levels are 1–50 nM, but these levels rapidly rise to more than 1 μM with tissue ischemia, hypoxia, or inflammation (Rivkees et al., 2001; Conway et al., 2003).

Adenosine and adenosine receptors influence a number of cellular processes, as well. For example, adenosine receptors activate transcription factors, e.g. NF-κB that in turn activates pro-inflammatory molecules (Eltzschig et al., 2004). Adenosine also plays a role in regulating cellular events by influencing the expression of the transcription factor Hypoxia Inducible Factor (HIF-1) (Eltzschig et al., 2004).

3. Adenosine receptors

Fluctuations in adenosine levels are sensed by transmembrane receptors that transduce adenosine's biological effects. There are two major classes of purine receptors - P1 and P2 Fredholm, 2010, Abbracchio et al., 2009. ATP and ADP bind to P2 purine receptors that include P2Y purine metabotropic receptors that couple with G-proteins (Abbracchio et al., 2009). P2 receptors also include the P2X receptors that are ion channels (Abbracchio et al., 2009).

Adenosine receptors (ARs) are P1 purine receptors (Fredholm, 2010; Fredholm et al., 2000, 2001). Similar to other G protein-coupled receptors (GPCRs), adenosine receptors contain seven putative transmembrane (TM) spanning domains (Fredholm, 2010; Fredholm et al., 2000, 2001). Adenosine receptors were initially cloned as orphan receptors (Libert et al., 1989). The identities of the genes encoding the A2a, A1, A2b, and A3 adenosine receptors were subsequently established in sequential order (Maenhaut et al., 1990; Libert et al., 1991; Rivkees and Reppert, 1992; Fink et al., 1992; Reppert et al., 1991; Zhou et al., 1992).

A1 and A3ARs activate $G_{i/o}$ protein which inhibits adenylyl cyclase and leads to decreased levels of cAMP (Fredholm et al., 2001). Alternatively, A2a and A2bARs activate G_s protein which stimulates adenylyl cyclase and leads to increased levels of cAMP (Fredholm et al., 2001). In addition, A1ARs activate phospholipase C, and open ion channels such as calcium channels (Fredholm et al., 2001).

Each adenosine receptor subtype has a different pattern of tissue expression and ligand binding properties. In cell-based systems, A1ARs have the highest affinity for adenosine (K_i 10 nM) (Fredholm, 2010; Fredholm et al., 2000, 2001). The K_i values for adenosine for the A2a, A2b and A3 adenosine receptors are 200, 2000, and 10,000 nM, respectively, for the human receptors (Fredholm, 2010; Fredholm et al., 2000, 2001). A3ARs are also activated by the adenosine metabolite inosine (K_i 2 300 nM) (Fredholm, 2010; Fredholm et al., 2000, 2001). Methylxanthines, including caffeine and theophylline, are nonselective adenosine receptor antagonists found in commonly consumed beverages including coffee and tea (Trivedi et al., 1990).

Highest levels of A1AR gene expression are detected in adult brain, fat, and testis (Reppert et al., 1991). Less prominent A1AR expression is seen in the heart and kidneys (Reppert et al., 1991). A2aAR gene expression is seen in brain, heart, and lung (Fink et al., 1992). A2bAR mRNA expression is highest in colon and bladder (Stehle et al., 1992). A2bARs expression is also high in retina (Blazynski, 1993). A3AR is expressed in testis, heart, and retina (Zhou et al., 1992). For A1 and A2aARs, the levels of gene and binding site expression are proportional, but for A3ARs gene expression is much greater than binding site expression (Rivkees et al., 2000).

In the brain, A2aARs are expressed in several brain regions, and heavy expression is seen in the striatum on cells expressing D2 dopamine receptors, an observation that dates back two decades (Fink et al., 1992). A2bAR expression is localized to the pars tuberalis region of the hypophysis (Rivkees and Reppert, 1992; Stehle et al., 1992). Functional studies have suggested the presence of A3ARs in the central nervous system (Lopes et al., 2003). A1ARs are among the most widespread GPCRs in the brain. In comparison with the relatively discrete expression of other receptor subtypes, A1AR expression is at high level throughout the brain (Reppert et al., 1991; Swanson et al., 1995).

In the heart, A1AR expression is present in atria and ventricles, and atrial A1AR expression is greater than that seen in the ventricles (Rivkees, 1995). A2aARs are present in coronary vessels in endothelial cells, smooth muscle cells of blood vessels and on myocytes (Olanrewaju et al., 2000). A3ARs are present in myocardial tissue, although at low levels (Zhou et al., 1992). A2bARs are present on endothelial cells, smooth muscles cells and fibroblasts (Eckle et al., 2008). Adenosine receptors are thus localized at sites to modulate cardiovascular system function.

4. A1ARs protect the embryo from hypoxic stress

A1ARs are the earliest expressed adenosine receptors in the fetal heart (Rivkees, 1995), and we demonstrated that cardiac A1AR expression protects the embryo from hypoxic insults (Wendler et al., 2007, 2010). It is likely that other adenosine receptor subtypes play important and possibly protective roles during development, however we have focused on the role of A1ARs in this report.

Because adenosine and A1ARs mediate adverse effects of hypoxia on the developing postnatal mammalian brain and lung (Turner et al., 2003), we anticipated that blockade of adenosine action would protect embryos from hypoxia (Turner et al., 2003). To our surprise, we observed that adenosine acting through A1ARs exerts dramatic protective effects during mammalian embryogenesis in response to hypoxic insults (Wendler et al., 2007, 2010).

Using a global A1AR knockout mouse model, we observed normal embryogenesis under normoxic conditions (Wendler et al., 2007). However, embryos lacking A1ARs were significantly more growth retarded under hypoxic conditions compared to embryos expressing A1ARs (Wendler et al., 2007). These data show that adenosine acting via A1ARs play an important role in protecting the embryo from hypoxia.