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PERSPECTIVE

The Thrill Can Kill: Murder by Methylation

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ABSTRACT

Why babies of crack-cocaine mothers develop heart problems has always been a mystery. In this issue of *Molecular Pharmacology*, Zhang et al. (p. 1319) show that a specific methylation occurs at the protein kinase Cε (PKCε) promoter of the babies born of mother rats exposed to cocaine. This reduces the expression of PKCε, a naturally cardioprotective enzyme, which provides a plausible molecular mechanism for cardiac failure.

The rampant use of cocaine as a recreational drug in the 1980s prompted the anticocaine commercial "The thrill can kill." The mood-enhancing effect of cocaine in the user is swift and fleeting. In contrast, the unfortunate children of crack cocaine mothers often inherit developmental and psychological disorders, such as low birth weight and learning disabilities (Bauer et al., 2005). They also show a higher incidence of heart failure and ischemic injury in adulthood. Although its exact cause remains unknown, a article in this issue of *Molecular Pharmacology* by Zhang et al. (2007) suggests a provocative molecular mechanism. These authors administered cocaine to pregnant laboratory rats and studied their offspring. Compared with pups of control rats not exposed to cocaine, those of cocaine-exposed mothers showed much lower cardiac levels of protein kinase Cε (PKCε), an enzyme known for its cardioprotective power. The mRNA level of PKCε was reduced even more dramatically. We were surprised to find that the nucleotide sequence of the PKCε promoter was unchanged, pointing to a possible "epigenetic" mechanism, defined by heritable changes in gene regulation without a change in DNA sequence. The search eventually led to a methylation event in which a methyl group placed on a sequence near the PKCε promoter prevented binding of the essential transcription factor activator protein-1 (AP-1), resulting in abrogation of PKCε transcription. It is clear that the specific methylation in the fetal genome, triggered by the mother's use of cocaine, led to the loss of PKCε expression, resulting in cardiac hypertrophy and susceptibility to ischemia and reperfusion injury later in life (Fig. 1). These results have important implications in the etiology and management of crack-cocaine children.

Cocaine, an alkaloid, prepared from the leaves of the plant *Erythroxylon coca* and first isolated by the German chemist Friedrich Gaedcke in 1855, has a long history of both medicinal and recreational use in the human society (http://en.wikipedia.org/wiki/Cocaine). World annual cocaine use currently stands at around 600 metric tons, with Americans consuming roughly half and Europeans, about a quarter. In its various forms (mainly "freebase" and "crack") cocaine is second only to cannabis as the most popular illegal recreational drug in the United States, amounting to an annual street business of approximately $40 billion. Like most psychostimulants and antidepressants, cocaine causes transient euphoria through the relatively well documented biochemical stimulation of the dopaminergic system. In contrast, little is known about the mechanism of the more lasting and inheritable effects of cocaine, which makes the studies of Zhang et al. (2007) significant.

Cocaine kills thousands of its users every year. Within minutes of administration, cocaine is found in a variety of organs, including brain, lung, kidney, and heart (Benveniste et al., 2005). At the clinical and physiological levels, the most common cardiac disorders are ischemia, acute coronary syndrome, myocarditis, cardiomyopathy, and arrhythmias. Past studies have revealed some effects of cocaine at the genetic level in the heart tissue, but their exact clinical correlations

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ABBREVIATIONS: PKC, protein kinase C; AP-1, activator protein-1.

await further study. These include reduction of sarcoplasmic reticular Ca^{2+}-ATPase (SERCA2a) levels (Wang et al., 2002a), induction of various forms of cytochrome P450 proteins (Wang et al., 2002b), and transcriptional up-regulation of harmful complements, possibly by reactive oxygen species (Tanhehco et al., 2000). In recent studies, the Zhang group also showed that prenatal cocaine exposure increased apoptosis or programmed cell death in neonatal rat heart and increased the susceptibility to ischemia-reperfusion injury in month-old rats (Bae and Zhang, 2005; Li et al., 2005). What sets the current study (Zhang et al., 2007) apart is its ability to offer an in-depth molecular mechanism based on known premises.

A major strength of the study derives from its focus on the offspring rather than the cocaine-using mother and the demonstration of a long-lasting epigenetic effect. The key molecular event is the specific methylation in a non–CpG-island region that normally functions as the enhancer for AP-1 binding. The abrogation of binding was demonstrated in a direct assay in vitro as well as by immunoprecipitation analysis of chromosomes in vivo. A number of control experiments were in place. For example, cocaine showed no effect on the binding of AP-1 on unmethylated PKCε enhancer sequence, showing that AP-1 itself was unaffected. Reporter gene assays provided in vivo confirmation of the loss of AP-1-dependent transcription when the methylated enhancer was used. Enhancers of a number of other transcription factors were not methylated, ruling out their role. Finally, methylation of the promoter region of another PKC (i.e., PKCδ) was unaffected. Although not specifically shown in this article, there is a large volume of literature on the cardioprotective role of PKCε that probably operates via phosphorylation of multiple downstream molecules (Liu et al., 1999; Inagaki et al., 2006; Morrow et al., 2006; Yue et al., 2006).

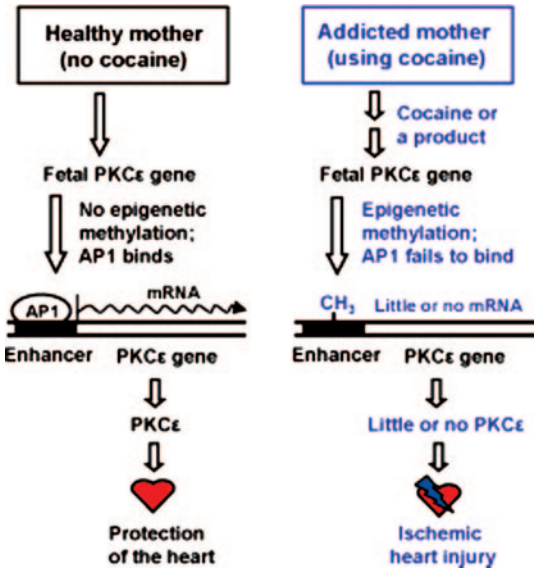
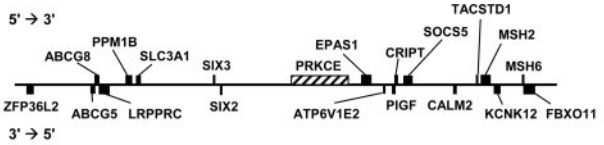


Fig. 1. Mechanism of fetal PKCε gene regulation by maternal cocaine use. The model is based on the findings of Zhang et al. (2007). Left, normal, drug-free mother. Right, mother addicted to cocaine, with the cocaine-related changes in blue color. In the absence of cocaine, the unmodified AP-1 enhancer site adjacent to the PKCε gene promoter in the fetal heart binds AP-1 and the fetal PKCε gene is expressed, offering cardio-protection. As a result of cocaine use by the mother, the same site is methylated, preventing AP-1 binding and PKCε expression, leaving the child's heart vulnerable to future ischemic injuries.

As with any leading study, the article brings up as many questions as answers. An important question is whether the observed effect is due to cocaine in the fetal blood or to a secondary effector produced by the mother's system in response to cocaine, which then traveled to the fetus. Cocaine itself is known to freely traverse from the mother's bloodstream to the fetal circulation (Benveniste et al., 2005), and the same is possible for other small molecule effectors. Because the placental transport cannot be obstructed without harming the fetus, a better therapeutic target may be the putative methyltransferase activity itself, which needs to be characterized. A relatively unique activity with specialized regulatory roles will be a desirable drug target for short-term intervention during each episode of cocaine use. Another question is the stability of the methylation through adulthood and old age. Zhang et al. (2007) correctly speculate that as the cardiomyocytes do not divide after birth the acquired methylation is likely to persist. However, this should be tested at various ages because methylation can be a reversible process.

The article also leaves the door open as to what activates the methylation in the first place. By far the two most common mechanisms of epigenetics are post-translational modifications of histone (e.g., acetylation, phosphorylation) and



Gene symbol	High expression in	Domains, description, function (putative)
ZFP36L2	Cardiac, hematopoietic	C3H1-type Zn finger; transcription factor
ABCG5	Vascular, digestive	ATP-binding cassette; ABC transporter; TM6
ABCG8	Similar to ABCG5 above	
LRCPPRC	Cardiovascular, hematopoietic, hepatic	Leu-rich PPR-motif; deficiency causes Leigh syndrome - French-Canadian type (LSFC) with developmental delay, COX deficiency etc.
PPM1B	Cardiovascular	Protein phosphatase 1B (PP2C)
SLC3A1	Intestine, neuroendocrine	Solute carrier family 3; amino acid transport; deficiency causes amino acid excretion and renal obstruction.
SIX3	Epithelial cells, fetal eye	Six domain, HLH homeodomain, deficiency causes holoprosencephaly 2 with cleft palate and lip, microcephaly, mental retardation.
SIX2	Neural, respiratory	Same family as SIX3; similar domains; transcription factor with role in embryogenesis and limb development.
PRKCE	Hippocampus, placenta	PKCε; Zn-dependent, phorbol ester- and DAG-binding Domains; role in signaling.
EPAS1	Cardiovascular, respiratory, placenta	Two PAS dimerization domains; bHLH transcription factor; hypoxia-inducible factor.
ATP6V1E2	Cardiovascular, respiratory, digestive	H^+-transporting ATPase; important in pregnancy, fetal optic nerve development.
PIGF	Blood / hematopoietic	Phosphotidylinositol glycan, class F; TM6; GPI-anchor.
CRIPT	Brain, heart	Cys-rich interactor of PDZ-three; Ubiquitin-activating motif; Postsynaptic protein; signaling neurotransmitter.
SOCS5	Hematopoietic, cardiovascular, neural	Suppressor of cytokine signaling 5; SH2 and CH-domains.
CALM2	Testis, colon, lung	EPS-homology and EF-hand domains; Calmodulin 2
TACSTD1	Epithelial	Tumor-associated calcium signal transducer 1; role in cell adhesion; carcinoma-associated.
MSH2	Lymph, testis	HLH domain associated with adenine nucleotide; mutator, mismatch repair, anti-oncogene.
KCNK12	Lymph, brain	Potassium channel.
FBXO11	Lymph, brain, testis, lung	"F-box only 11"; UBR-type Zn-finger; transcription factor.

Fig. 2. The human genomic PKCε locus. Top, location of PKCε (PRKCE) and its neighbor genes on chromosome 2p21 based on GENATLAS (http://www.dsi.univ-paris5.fr/genatlas/). Bottom, short description of the same genes, including the tissue(s) in which they are expressed abundantly, their major domains, and known or putative functions.

methylation of DNA. Both play important roles in a variety of inheritable phenomena in health and disease, including tissue-specific and parental imprinting, X-chromosome inactivation, development and cancer (Couture and Trievel, 2006; Wood and Oakey, 2006). It would be interesting to determine whether histone modification precedes and actually regulates the accessibility of the DNA to the methylation process discovered by Zhang et al. (2007). Pioneering studies have revealed that sets of adjacent genes are often regulated jointly, most likely through ultrastructural remodeling of the chromosome, mediated by local modification of histone (Spellman and Rubin, 2002). Each set may cover 20 to 200 kilobase pairs, encompassing 10 to 30 genes. Although the full physiological implication of this finding remains to be determined, an examination of the genes on both sides of the PKCε gene on chromosome 2p21 reveals that they code for critical signaling proteins, such as transcription factors, phosphatases, and transporters (Fig. 2). These genes are highly expressed in cardiovascular as well as in hematopoietic, digestive, retinal, neural, and reproductive tissues. Indeed, it is probably not a coincidence that significant damage to all these tissues is observed in cocaine users (Summavielle et al., 2000; Darland and Dowling, 2001; Li et al., 2003; Wilson and Saukkonen, 2004; Ellis and McAlexander, 2005; He et al., 2006). In light of the findings of Zhang et al. (2007), it can now be tested whether the PKCε-neighboring genes are also regulated in the cocaine-exposed children, positively or negatively. Finally, small noncoding RNAs such as microRNAs, transcribed from RNA PolII promoters, are now known to regulate a variety of cellular processes, including chromatin methylation and silencing (Almeida and Allshire, 2005; Barik, 2005). If a specific noncoding transcript plays a role in altering the chromatin structure for this particular methylation or for other epigenetic fetal changes, it will open new layers of regulation. Recent studies have indeed shown that altered miRNA levels may underlie disease states and that there seems to be a correlation between locations of miRNA genes with sites of genetic alteration (Calin and Croce, 2006; Huppi et al., 2007). Regardless of the mechanism, it seems that we are witnessing the dawn of a new era in which extensive studies of fetal epigenetic changes will be undertaken. Comprehensive genome-wide analyses may unravel the epigenetic signature of each intrauterine and/or environmental factor, which will affect both basic and clinical sciences.

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