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Caffeine induction of *Cyp6a2* and *Cyp6a8* genes of *Drosophila melanogaster* is modulated by cAMP and D-JUN protein levels

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ABSTRACT

Cytochrome *P450* monooxygenases or CYPs, a family of endobiotics and xenobiotics metabolizing enzymes, are found in all organisms. We reported earlier that the promoters of *Drosophila Cyp6a2* and *Cyp6a8* genes are induced by caffeine. Since caffeine antagonizes adenosine receptor (AdoR) and inhibits cAMP phosphodiesterase (PDE), we used *luciferase* reporter gene to examine whether in SL-2 cells and adult *Drosophila*, induction of the two *Cyp6* genes is mediated *via* AdoR and/or PDE pathway. Results showed that AdoR is not involved because AdoR agonists or antagonists do not affect the *Cyp6* promoter activities. However, inhibition of PDE by specific inhibitors including caffeine causes induction of both *Cyp6* gene promoters. We also found that flies mutant for *dunce* gene coding for cAMP-PDE, have higher *Cyp6a8* promoter activity than the wild-type flies. We demonstrate that caffeine treatment increases intracellular cAMP levels, and cAMP treatment induces the *Cyp6* gene promoters. Since both *Cyp6* genes have multiple sites for JUN transcription factors, which generally play a positive role in cAMP pathway, effect of *Drosophila jun* (*D-jun*) on the *Cyp6a8* promoter activity was examined. Results showed that the expression of D-jun sense plasmid causes downregulation rather than activation of the *Cyp6a8* promoter. Conversely, expression of antisense plasmid increased the promoter activity. Interestingly, caffeine treatment decreased the D-JUN protein level in SL-2 cells as well as in adult flies. These results suggest that D-jun acts as a negative regulator, and caffeine induction of *Cyp6a8* and *Cyp6a2* genes is mediated by the upregulation of cAMP pathway and downregulation of the D-JUN protein level.

1. Introduction

Caffeine, the most commonly used psychostimulant, is found in coffee, tea, many soft drinks and several drug preparations. Because of its widespread use, the effect of caffeine on various physiological processes has been studied extensively and mostly in humans, rats, mice and other mammals (Svenningsson et al., 1995a; Lorist and Tops, 2003; Porta et al., 2003). Comparatively, very little is known about the effect of caffeine in insects. In *Drosophila melanogaster*, caffeine treatment has been shown to cause chromosome loss in the larvae and lethality to the adult flies (Clark and Clark, 1968; Zimmering et al., 1977). In *Drosophila prosaltans*, low dose of caffeine treatment decreases both fecundity and longevity (Itoyama et al., 1998). Caffeine that is naturally found in plants inhibits insect feeding and acts as a pesticide (Nathanson, 1984). Caffeine is also found to be effective in killing or repelling slugs and snails when applied to foliage (Hollingsworth et al., 2002). When adult *Drosophila* are allowed to feed on caffeine containing food, a dose-dependent decrease in rest was observed (Shaw et al., 2000). Interestingly, during normal wake or active period, the expression of *Cyp4e2* gene was found to be increased about 2-fold higher compared to the rest or sleep period (Shaw et al., 2000).

A modest number of studies have also been done to investigate the effect of caffeine on gene expression. In one study, caffeine treatment has been shown to increase CYP1A1 and CYP1A2 mRNA levels in rat liver and kidney (Goasduff et al., 1996). In other studies, caffeine was found to increase the levels of c-Fos, c-Jun, and junB mRNAs in rat striatum (Svenningsson et al., 1995b), and sonic hedgehog mRNA in primary murine neuronal and astroglial cells in culture (Sahir et al., 2004). We also reported that caffeine increases the *Cyp6a2* and *Cyp6a8* promoter activity both in adult females and in SL-2 cells. The 0.98- and 0.8-kb upstream DNA of *Drosophila Cyp6a2* and *Cyp6a8* genes respectively, have *cis*-regulatory elements that are involved in high level of caffeine induction (Bhaskara et al., 2006). In a recent study, caffeine treatment has been shown to increase the levels of eleven *Cyp* gene transcripts in *Drosophila* larvae (Willoughby et al.,

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Abbreviations: AdoR, adenosine receptor; bp, base pair(s); cAMP, 3′,5′ cyclic adenosine monophosphate; cAMP-PDE, cAMP phosphodiesterase; CYP, cytochrome *P450* monooxygenase; *Cyp* (or *CYP*), CYP-encoding gene; DDT, kb, kilobase(s) or 1000 bp; nt, nucleotide(s); LUC, Luciferase; *luc*, LUC-encoding gene; nt, nucleotide(s); PDE, phosphodiesterase.

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2006). Although these studies demonstrate that caffeine can induce the transcription of *Cyp* genes, the underlying mechanism of the induction process is not known.

In mammals, caffeine exerts its action by antagonizing the A_1 and A_{2A} isoforms of adenosine receptor (AdoR) and/or by inhibiting the cAMP phosphodiesterase enzyme (PDE) (Poulsen and Quinn, 1998; Fredholm et al., 1999; Fisone et al., 2004). Antagonism of adenosine receptor or PDE inhibition is known to elevate the intracellular cAMP level and activation of cAMP-mediated pathway has been shown to induce the expression of activator protein-1 (AP-1) family transcription factors including c-Jun and c-Fos proteins (Karin, 1995a; Fredholm et al., 1999). In fact, caffeine has been shown to increase the expression of c-Fos, c-Jun, and junB mRNAs or protein in the rat brain striatum (Svenningsson et al., 1995b; Bennett and Semba, 1998a,b). Sequence analysis of the caffeine-responsive 0.8- and 0.98-kb upstream DNA of the *Cyp6a8* and *Cyp6a2* genes, respectively, has identified the presence of several putative AP1 binding motifs (Bhaskara et al., 2006). It is possible that caffeine induction of *Cyp6a2* and *Cyp6a8* genes may be mediated *via* the signaling pathways described above and the putative AP1 binding motifs. To test these possibilities and to understand the mechanism of caffeine-mediated transcriptional activation, we examined the effects of various antagonists and agonists of AdoR and PDE on the *Cyp6a2* and *Cyp6a8* gene promoter activities in SL-2 cells as well as in the transgenic flies. We also examined the effects of cAMP and *Drosophila* Jun protein (D-jun) on *Cyp* promoter activity. Our results show that caffeine induces *Cyp6a2* and *Cyp6a8* genes by activating the cAMP pathway and by downregulating the D-jun protein level. Data show that D-jun acts as a negative rather than a positive regulator for the expression of *Cyp6* genes.

2. Materials and methods

2.1. Fly stocks, cell culture and chemicals

Two reporter transgenic lines of *D. melanogaster*, *0.2-luc4H-ry* and *0.8-luc110H-ry*, dnc^1 mutant strain and Schneider line SL-2 cells (Schneider, 1972) were used in the present investigation. The dnc^1 mutant strain was obtained from Indrani Ganguly of Neuroscience laboratory, San Diego (CA). The SL-2 cells were obtained from Invitrogen (Carlsbad, CA) and maintained at 25 °C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated bovine calf serum and 0.01% penicillin–streptomycin (Sigma, St. Louis, MO). Every fourth day, the cells were transferred to fresh media. The reporter transgenic lines were homozygous for single copy of *0.2-luc* or *0.8-luc* reporter transgene located on the second chromosome in ry^{506} background (Maitra et al., 2002; Bhaskara et al., 2006). These transgenes were created by cloning a 0.2- (−11/−199) or 0.8- (−11/−761) kb upstream DNA of *Cyp6a8* gene of the DDT resistant 91-R strain in front of the firefly *luciferase* (*luc*) gene. The details of the construction of reporter plasmids and germ line transformation into ry^{506} host strain have been previously described (Maitra et al., 2002). All flies were raised on standard corn meal–agar–molasses medium and maintained at 25 °C under 12 h dark–light cycle.

Caffeine, adenosine, 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), N^6 cyclopentyl xanthine (CPA), theophylline, rolipram and dibutyryl cAMP were purchased from Sigma (St. Louis, MO). Caffeine, theophylline and dibutyryl cAMP were dissolved in sterile water, whereas CPA, DPCPX and rolipram were dissolved in DMSO. Adenosine was dissolved in 250 mM Tris pH 8.0.

2.2. Construction of reporter plasmids

To create 0.9luc-A2 chimeric reporter plasmid, a 0.9-kb upstream DNA of *Cyp6a2* between positions −983 and −1 (ATG at +1) was amplified by PCR using the following primer pairs: 5′-ctcacgcgtTTCATTCGTTTTATCGCCG-3′ (−983/−965) and 5′ctcgtcgacTTTGCGTAGCTGCTCCC-3′ (−1/−17). The sequences shown in lower cases at the 5′-ends of the distal (−983/−965) and proximal (−1/−17) primers, have engineered sites for MluI (acgcgt) and SalI (gtcgac) restriction enzymes. The PCR product was digested with MluI and SalI, and cloned in front of the *luc* (*luciferase*) reporter gene between the MluI and XhoI sites of the pGL3-Basic vector (Promega, Madison, WI). Construction of 0.2luc-A8 and 0.8luc-A8 reporter plasmids have been described previously (Maitra et al., 2002). All cloned PCR products were verified by sequencing at the Molecular Biology Resource Facility of the University of Tennessee.

2.3. Construction of D-jun promoter construct

The organization of the *D-jun* gene of *D. melanogaster* has been described previously (Wang and Goldstein, 1994; Rousseau and Goldstein, 2001). Based on this report, and *Drosophila* genome database, two PCR primers, D-jun-up (−540) and D-jun-up (+1), were designed to amplify the −540/+1 DNA of the *D-jun* gene. The sequences of these distal and proximal upstream DNA primers respectively were as follows: g*gacgcgt*TCCTTTCCTATTTACCGACGCC and g*gctcgag*GGGTGGGAACTTTG. The MluI and XhoI sites added to the 5′-ends of the distal and proximal primers, respectively, are shown in lower cases. These primers span −540 to +1 region of the *D-jun* gene corresponding to the bases between 248772 and 249271 regions of the *Drosophila* genomic scaffold. Genomic DNA isolated from *0.8luc110H-ry* reporter transgenic line was used as a template to amplify the upstream DNA of *D-jun* gene *via* PCR. The amplified DNA obtained for each gene was cloned into pGEMT-Easy vector (Promega, Madison, WI) and sequenced. Using the BLAST program, the sequences were compared with the upstream DNA sequences reported in the database for each gene. The PCR products were then excised by cutting with MluI and XhoI, and cloned into MluI/XhoI cut pGL2 (N) basic vector, which is the pGL2-basic vector (Promega, WI) with SalI site modified to NotI (Maitra et al., 2002).

2.4. Construction of D-jun expression plasmids

For the D-jun plasmid construction, several plasmids were obtained from different sources. D-jun/pBSK-carrying full-length D-jun with 6× his-tag at the C-terminal end was kindly provided by Dirk Bohmann, University of Rochester. FJF/pCasper4 plasmid carrying tubulin (Tub) promoter was obtained from Laura Ciapponi, EMBL, Heidelberg. D-jun/HS plasmid carrying D-jun cDNA in sense (S) (D-jun (S)/HS) or antisense (AS) (D-jun (AS)/HS) orientations driven by Hsp70 promoter in pCasper plasmid was obtained from Nobert Perrimon, Harvard University.

To create the D-jun cDNA plasmid in sense and antisense orientation, the FJF/pCasper4 plasmid carrying tubulin (Tub) promoter was first modified. This plasmid has unique KpnI and XbaI sites that flank the FJF insert (Ciapponi et al., 2001). To be able to remove the FJF insert as an XbaI fragment, the plasmid was cut with KpnI and the 3′-overhang was removed by digesting with S1 nuclease. The blunt-ended plasmid was then ligated with XbaI linker (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The converted plasmid was then used to transform *DH5α E. coli* bacteria. The plasmid was purified and the FJF insert was removed by cutting with XbaI. The remaining part of the plasmid was then self-ligated to create pCasper4-Tub vector with a unique XbaI site downstream of the tubulin promoter. To isolate the D-jun cDNA as an XbaI fragment, the D-jun/pBSK-plasmid was used. This plasmid has D-jun cDNA flanked by a unique XbaI site at the 5′-end and a HindIII site at the 3′-end. The plasmid was cut with HindIII and the 5′-overhangs were repaired by Klenow polymerase. The resulting blunt-ended plasmid was the ligated with XbaI linker used to transform *DH5α E. coli* bacteria. The purified plasmid was cut with XbaI, the D-