Digoxin and Genistein Treatment by *Drosophila* Larvae Differentially Modulate the Levels of Arginine Kinase Encoding Transcripts

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Abstract

Drosophila melanogaster arginine kinase (Argk) gene encodes 6 different mRNA transcripts: Argk-RA to Argk-RF, due to alternative promoters and splicing. To examine the impact of digoxin and genistein on *Drosophila*, total mRNA was extracted from 3rd instar larvae after chemical treatment and the level of different transcripts were tested by RT-PCR. Result demonstrates that both digoxin and genistein can up-regulate the level of Argk transcripts in general. Argk-RC is up-regulated by both chemicals whereas Argk-RA, Argk-RD and Argk-RE have no significant changes. This finding indicates that digoxin and genistein can differentially influence the utilization of arginine kinase gene promoters.

Introduction

Arginine kinase (AK; 2.7.3.3) belongs to the family of phosphagen kinases (PK) or guanidino kinases. AK is the only PK of most invertebrates and is the primary enzyme in cellular energy metabolism and ATP-consuming processes in invertebrates. The common PK of mammals is creatine kinase. Both the reactive domains in arginine kinase and creatine kinase are highly conserved.

Arginine kinase catalyzes the chemical reaction:

$$\text{Mg-ATP + L-arginine} \overset{\text{AK}}{\longleftrightarrow} \text{Mg-ADP + } H^+ \text{ + N-}\omega\text{-phospho-L-arginine}$$

Arginine kinase buffers cellular ATP and provides the dynamic energy homeostasis of high energy consuming cells and tissues. AK has a relatively high expression in brain, muscle, gonads and imaginal disc.

Arginine kinase gene (Argk) is mostly studied in Mollusca and Arthropod. Six transcripts (Argk-RA to Argk-RF) can be produced by *Drosophila melanogaster* Argk gene (Figure 1) through alternative promoters and splicing. This is a very unique pattern compared with Argk genes in other invertebrate and homologous creatine kinase genes in most of vertebrate. In other invertebrate, Argk always produces one or two transcripts, whereas in vertebrate, different creatine kinase isoforms are always encoded by different creatine kinase genes.

All of the transcripts share 3' exon7 but differ with unique 5' exons. Argk-RA and Argk-RE are predicted to be generated by alternative splicing because Argk-RE splices exon4 compared with Argk-RA. Recent study identified exon4 in

Argk-RA as a signal for mitochondria tranportation. Argk-RD and Argk-RF, with an extra 86 bp sequence resulting form 3' of exon6, are also presumed to be alternative spliced isoforms. Only five different arginine kinases can be produced by different start codon selection because Argk-PD (protein of Argk-RD) and Argk-PF (protein of Argk-RF) use same start codon and stop codon in exon7. Exon7 encodes a carboxyl terminal domain for substrate binding and catalyzing reaction and other exons encode different amino terminal regulatory domains of arginine kinases.

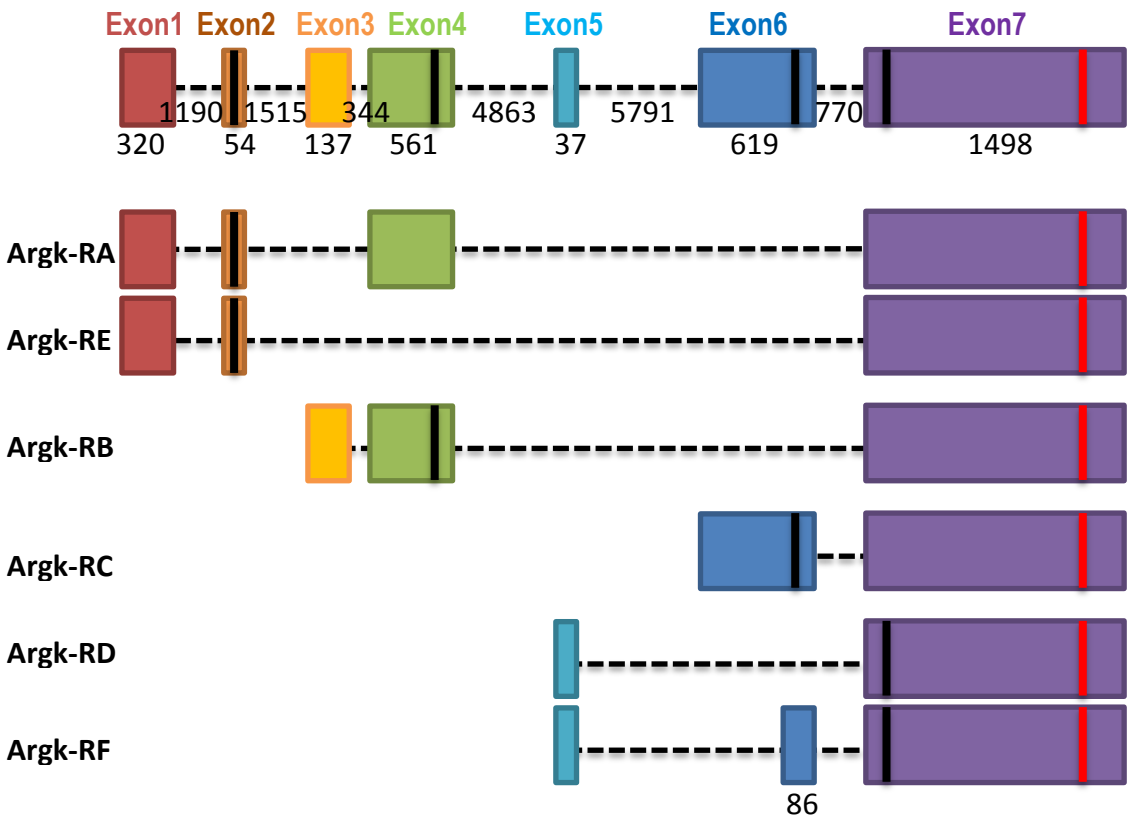

Figure 1. Six putative transcripts of *Drosophila melanogaster* arginine kinase gene from Flybase database (Annotation ID: G32031).

It is predicted there are four promoters of Argk gene. One promoter regulates the expression of Argk-RA and Argk-RE and another one promoter regulates the expression of Argk-RD and Argk-RF. Argk-RB and Argk-RC are regulated by the other two promoters individually.

Expression profiles have shown that Argk has two peaks during 3rd instar larvae and pre-eclosion stage indicating Argk is developmental stage related gene but stage or tissue specific expression patterns of each of the six isoforms are still unclear. Several flightless mutants in *Drosophila* can change AK activity and distribution.

No studies on the function of different Argk isoforms in *Drosophila* have been reported. Identification of compounds that differentially regulate the expression of specific Argk isoform will be a valuable tool for studying functions of individual Argk isoforms.

Digoxin, a cardiac glycoside to treat with heart failure or irregular heart rhythm, is an inhibitor of sodium-potassium ATPase. In heart muscle cell, high concentration of calcium is then produced to generate strong heart beats and other signaling pathway change. Genistein, an isoflavone from soy, can interact with several hormone or cytokine receptors, thus influencing the signaling pathway. Digoxin and genistein have been demonstrated to be capable of modulating mRNA expression.

To investigate the impact of digoxin and genistein on mRNA expression of Argk transcripts, RNA was isolated from 3rd instar larvae fed by digoxin and genistein and subjected to RT-PCR analysis.

Material and methods

Drosophila melanogaster and treatments

Drosophila 3rd instar larvae are used in this project. 3rd instar larvae were kindly provided by Dr. Dubrovsky's lab, Department of Biological Sciences, Fordham University. Different treatments are listed in Table 1.

#	Source	Treatment	Time
1	WT late 3rd	10 μl/g DMSO	14 hr
2	WT late 3rd	10 μg/g Digoxin	14 hr
3	WT late 3rd	100 μg/g Digoxin	14 hr
4	WT late 3rd	25 μg/g Genistein	14 hr
5	WT late 3rd	100 μg/g Genistein	14 hr

Table 1. 3rd instar larvae are fed by different digoxin and genistein concentrations for 14 hours

RNA extraction

Total RNA from different 3rd instar larvae treatment was extracted and purified from the cell lysates using the RNeasy® Plus Mini Kit (QIAGEN) by manufacturer's instructions. RNA was eluded with 50μl of RNA free dH2O. Concentration of each RNA extraction was tested by Spectrophotometer. A 10 ng/μl dilute was made from each RNA stock and used in RT-PCR.

RT-PCR

RT PCR was performed using QIAGEN® One-Step RT PCR Kit following the instructions. Rp49 was used as the loading control. 20 ng of RNA were

amplified in 20 μl RT-PCR reactions (4 μl 5×RT buffer, 0.8 μl 10 mM dNTPs, 0.8 μl enzyme mix, 0.8 μl 10 pmol/μl forward primer, 0.8 μl 10 pmol/μl reverse primer, 2 μl 10 ng/μl RNA and 10.8 μl RNA free dH2O).RT-PCR programs are: Reverse transcription of 50°C for 30 min, HotStarTaq DNA Polymerase activation of 95°C for 15 min, 40 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 60 sec, then a final extension of 72°C for 10 min followed by holding at 4°C.

Gel Electrophoresis

4 μl 6X loading dye was added to each 20 μl RT-PCR product and 5 μl products were loaded on 1.0 % agarose gels (2.0 mg agarose, 200 ml 1X TBE, 20 μl ethidium bromide). The voltage of electrophoresis is 180 V. Gels were visualized in BioRad UV trans-illuminator and pictures were taken.

PCR product purification, gel extraction and sequencing

Argk-RC, RD and total Argk PCR products were purified using QIAquick® PCR Purification Kit following the manufactures instructions and Argk-RA and RE products were cut from gel and purified using QIAquick® Gel Extraction Kit following the manufacturer's instructions. Purified PCR products were sent out for sequencing.

Primers

Information for specific designed primers is listed in Table 2 and Figure 2 shows locations of each primer pairs.

Argk Isoform	Primer	Length	Sequence(5' to 3')	Location (Figure2)	Product Size
Argk-RA Argk-RE	REA-F	20	ACAATCGAGACCAGTCCTCG	exon1	885
	CR-R1	20	CGAAGACCTCCTTGGTCAGG	exon7	324
Argk-RB	RB-F	23	TTTATTGATTTTCAACGGTAGGC	exon3	720
	CR-R1	20	CGAAGACCTCCTTGGTCAGG	exon7	
Argk-RC	RC-F	22	GTTGAATTCTACGGTTGCAAAG	exon6	304
	CR-R2	21	CAACTTGGCATAACCCTCCTC	exon7	
Argk-RD Argk-RF	RDF-F	22	GTTGAATTCTACGGTTGCAAAG	exon5	141
	CR-R1	20	CGAAGACCTCCTTGGTCAGG	exon7	227
Argk (all isoforms)	AK-F	20	AAGACCTTCCTGGTCTGGTG	exon7	329
	AK-R	20	ATGTCGTAGACACCACCCTC	exon7	
Rp49	Rp49-F	21	TCCTTCCAGCTTCAAGATGAC		464
	Rp49-R	21	GTGTATTCCGACCACGTTACA		

Table 2. RT-PCR primers for Argk-RA/RE, Argk-RB, Argk-RC, Argk-RD/RF, Argk(all isoforms) and Rp49

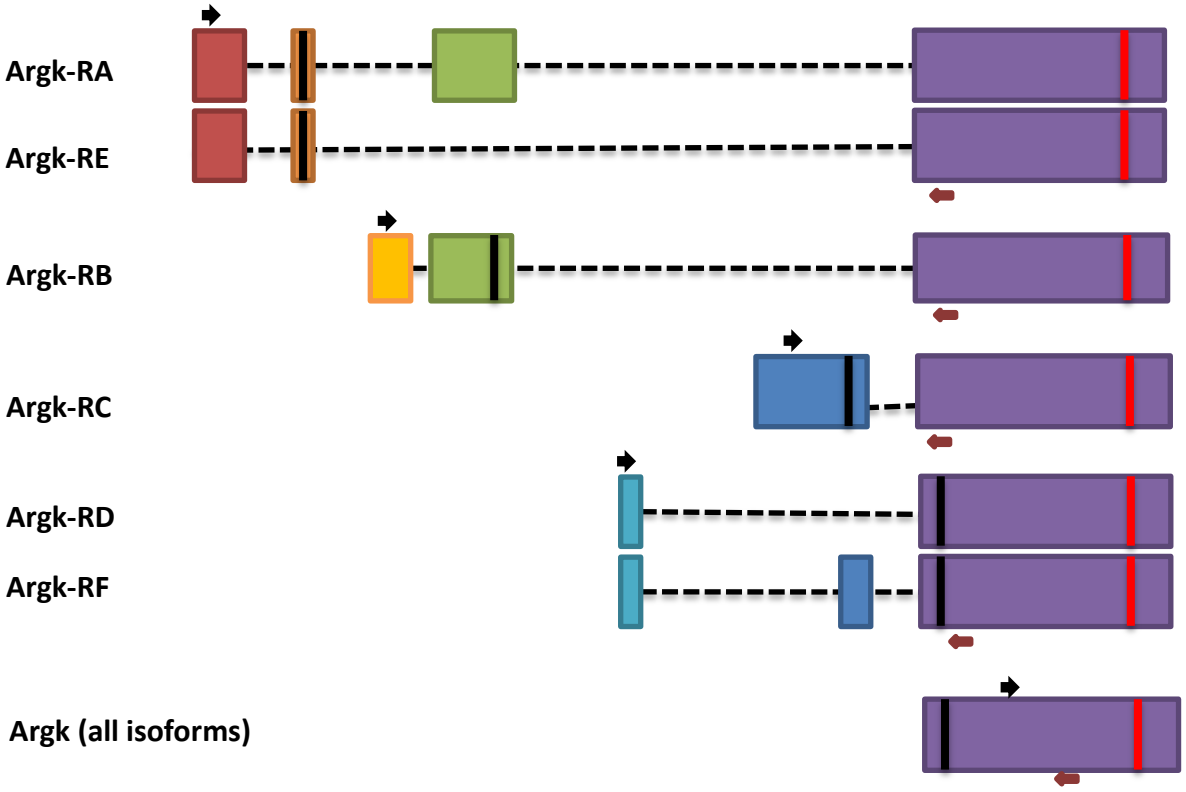

Figure 2. Location of primers for different Argk isoforms

Result

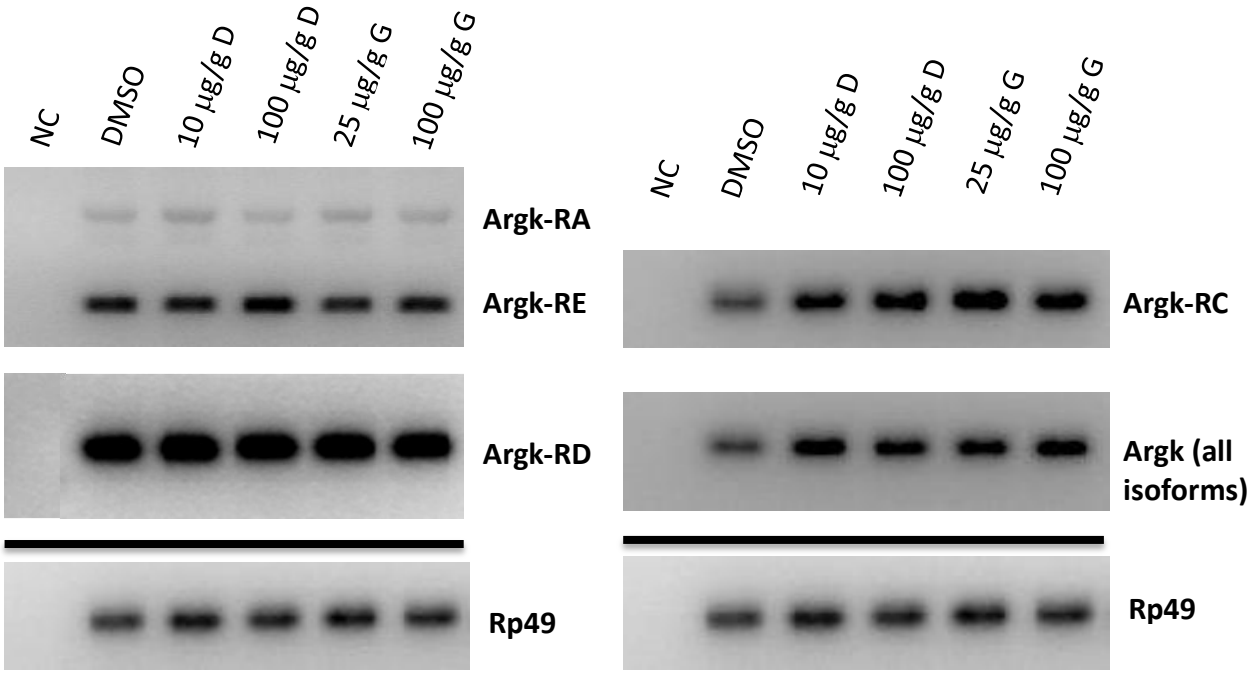

Figure 3. No significant mRNA expression differences have been observed with regard to Argk-RA, Argk-RE and Argk-RD by digoxin or genistein treatment. RT-PCR was performed on RNA isolated from *Drosophila* 3rd instar larvae fed with chemicals for 14 hours using primers specific to Argk-RA/RE and Argk-RD/RF. Treatments are DMSO (control), 10 μg/g digoxin, 100 μg/g digoxin, 25 μg/g genistein and 100 μg/g genistein from left to right. Rp49 is a loading control.

Figure 4. Argk-RC mRNA level is up-regulated by digoxin and genistein treatment and total Argk transcript level is also increased. RT-PCR was performed on RNA isolated from *Drosophila* 3rd instar larvae fed with chemicals for 14 hours using primers specific to Argk-RC and Argk all isoforms. Treatments are DMSO (control), 10 μg/g digoxin, 100 μg/g digoxin, 25 μg/g genistein and 100 μg/g genistein from left to right. Rp49 is a loading control.

Argk-RA, Argk-RC, Argk-RD and Argk-RE are expressed in *Drosophila* 3rd instar larvae, but Argk-RF is not detected in *Drosophila* 3rd instar larvae. Argk-RF and Argk-RD are designed to be picked up by same primer pairs so that Argk-RF is not expressed in the 3rd instar larvae. Results also indicate that digoxin or genistein treatment does not modulate mRNA expression of Argk-RA/RE or Argk-RD (Figure 3). Argk-RA and Argk-RE are alternative spliced transcripts and Argk-RA has an extra exon4 encoding a proline-rich mitochondria transport signal in the amino terminal of arginine kinase isoform A (Argk-PA). Result reveals that digoxin or genistein has no influence towards the Argk-RA/RE alternative splicing process.

Argk-RC mRNA level is up-regulated by digoxin and genistein treatment and total Argk transcripts also rise up in 3rd instar larvae (Figure 4). High concentration (100 μg/g) digoxin treated larvae has higher Argk-RC mRNA level than low concentration (10 μg/g) digoxin treated larvae. Both 25 μg/g and 100 μg/g genistein treated larvae have same level of total Argk mRNA higher than untreated larvae. Total six Argk isoforms are detected by a primer pair located in exon7 and PCR amplification of this primer pair has no products produced (result no shown) which confirms that Argk band in Figure 4 is produced only by mRNA.

Discussion

Digoxin and genistein treatment up-regulates mRNA level of Argk-RC of *Drosophila* 3rd instar larvae. Total Argk transcripts also increase in response to digoxin and genistein.

Digoxin or genistein treatment has no significant impact on mRNA expression levels of Argk-RA, Argk-RE or Argk-RD of *Drosophila* 3rd instar larvae. Meanwhile, Digoxin or genistein treatment has no significant impact on mRNA alternative splicing of Argk-RA and Argk-RE.

Argk-RF is not expressed in *Drosophila* 3rd instar larvae and expression of Argk-RF may be induced by hormones in other developmental stages.

Different transcripts of *Drosophila* Argk are produced by alternative promoters as well as alternative splicing. It is predicted that there are four alternative promoters in *Drosophila melanogaster* arginine kinase gene. Argk-RA and Argk-RE share one promoter; Argk-RD and Argk-RF share another promoter and Argk-RB and Argk-RC have one unique promoter for each.

Results in this project indicate that digoxin and genistein may differentially modulate the utilization of *Drosophila* Argk promoters. This phenomenon is probably due to different regulatory mechanisms of four promoters. Digoxin and genistein are capable of modulating Argk-RC promoter. Since no paper published mentions tissue or stage specific expression of Argk transctripts, the regulation mechanism remains to be determined.

Argk-RB expression has been detected by RT-PCR with no consistent result. Under same RT-PCR program, results range from no expression to light expression of all treated samples and also specific band showed up by some samples but not all (results not shown). There are two possible explanations for this result. One possibility is that Argk-RB expression is relatively low in 3rd instar larvae and RT-PCR detection is influenced mainly by initiating several amplifying cycles. The other possibility is low primer efficiency. Forward primer is designed in exon3 with 137 base pairs which has a very low GC% means that five consecutive A or T show up frequently. Indeed the forward primer designed for Argk-RB bears six consecutive A or T in 5' end and GGC in 3' end which are supposed to bad structures for primer functions.

Further work can continue by designing new primers to get consistent result of Argk-RB. Also, we can use real-time RT-PCR to get more accurate analysis to explain the amount of initial mRNA. Afterward, western can be used to detect protein level differences to gain more data of digoxin and genistein responses. Also we can test different transcripts using mRNA extracted from different developmental stages. Furthermore, other chemicals or even hormones can be tested to explore more about functions of different Argk transcripts.

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