Increased extracellular adenosine in Drosophila that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death

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SUMMARY
Extracellular adenosine is an important signaling molecule in neuromodulation, immunomodulation and hypoxia. Adenosine dysregulation can cause various pathologies, exemplified by a deficiency in adenosine deaminase in severe combined immunodeficiency. We have established a Drosophila model to study the effects of increased adenosine in vivo by mutating the main Drosophila adenosine deaminase-related growth factor (ADGF-A). Using a genetic screen, we show here that the increased extracellular adenosine in the adgf-a mutant is associated with hyperglycemia and impairment in energy storage. The adenosine works in this regard through the adenosine receptor as an anti-insulin hormone in parallel to adipokinetic hormone, a glucagon counterpart in flies. If not regulated properly, this action can lead to a loss of energy reserves (wasting) and death of the organism. Because adenosine signaling is associated with the immune response and the response to stress in general, our results mark extracellular adenosine as a good candidate signal involved in the wasting syndrome that accompanies various human pathologies.

INTRODUCTION
A genetic deficiency of adenosine deaminase (ADA) is accompanied by greatly elevated levels of its substrates adenosine and deoxyadenosine. This deficiency causes severe combined immunodeficiency (SCID) in both humans and mice, as well as pathological changes in other tissues (Blackburn et al., 1998; Hershfield and Mitchell, 2001). Elevated levels of extracellular adenosine are also observed during multiple organ failure (Hasko et al., 2002), the cause of 50-80% of all deaths in surgical intensive care units.

Adenosine is an endogenous purine nucleoside that has multiple functions. It is a structural component of nucleic acids, but it also plays an important role in physiological regulation both inside and outside the cell. Inside the cell, it acts as an intermediate in the biosynthetic pathway leading to ATP. However, it can be released from cells or formed extracellularly from ATP/ADP/AMP. The extracellular form plays an important role in controlling various processes, including neuromodulation (Dunwiddie and Masino, 2001), vasoconstriction and hormone release (Nyce, 1999). The release of adenosine in some tissues has been shown to be stimulated by hypoxia (Buck, 2004). Specific inflammatory stimuli, such as bacterial products, are also capable of triggering adenosine release from immune cells (Bodin and Burnstock, 1998). Adenosine regulates cellular functions by binding to G-protein-coupled adenosine receptors (A1, A2a, A2b and A3 in mammals) that can regulate intracellular cAMP (Latini and Pedata, 2001).

The role of adenosine as a signaling molecule is in many ways indistinguishable from that of a hormone and it is sometimes called a ‘stress hormone’ (Hasko et al., 2002). Compared with typical hormones, which are usually secreted from a storage pool or synthesized from a precursor in response to a specific external stimulus, adenosine formation is typically a consequence of the metabolic status within the secreting cell, as is the case for regulatory metabolites. Therefore, adenosine can be seen as a regulatory metabolite that has extended its sphere of action beyond the cell that generates it (Newby, 1985).

In animals, there are two groups of enzymes with adenosine deaminase activity, referred to as ADA1 and ADA2. Although the protein responsible for ADA1 activity, which when defective is associated with human SCID, has been known for long time (Valerio et al., 1984), the protein with ADA2 activity has only recently been isolated (Zavialov and Engstrom, 2005). ADA2 is encoded by human CECR1 gene, which is a member of a novel family of adenosine deaminase-related growth factors (ADGFs) (Maier et al., 2005).

We have established a Drosophila model to study the effects of increased levels of adenosine by targeting Drosophila ADGFs using homologous recombination mutagenesis (Dolezal et al., 2003). Six Drosophila ADGF genes are homologs of human CECR1 and thus belong to the family of ADA2 genes (Dolezelova et al., 2005). We showed that a product of the ADGF-A gene is a secreted, active adenosine deaminase (Zurovec et al., 2002) and that it is the major source of the ADA activity during Drosophila larval development (Dolezal et al., 2005).

The adgf-a mutation leads to dramatically increased levels of adenosine and deoxyadenosine in the larval hemolymph (insect blood) and to larval or pupal death, associated with fat body disintegration and formation of melanotic capsules in the late third-instar larvae (Dolezal et al., 2005). Melanotic capsule formation is accompanied by a massive release of hemocytes from the lymph gland. Both intracellular (by cell uptake) and extracellular (i.e. signaling) effects of the increased adenosine concentration might play roles in the adgf-a phenotype.
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G-protein subunits signaling similarly to mammalian A2B receptor by coupling to the (AdoR) (Dolezelova et al., 2007), which activates cAMP and calcium (Parks et al., 2004) (Fig. 1A). The deficiency spanned designated the PhK inserted in the second exon of an alternatively spliced variant (designated P{EP}PhKgamma[EP779] (Fig. 1A). We tried an available P element insertion suppression effect was very weak on a pure yeast diet and only when adgf-a mutant larvae on yeast (a protein-rich diet) supplemented with different amounts of carbohydrates. We found a striking sensitivity of the adgf-a mutants to the sugar content in the diet. When larvae were grown on a yeast diet supplemented with 5% sucrose they expressed the typical adgf-a mutant phenotype (Fig. 2A). However, when the mutant larvae were grown on a pure yeast diet without any additional sugar supplement, they all died during the larval stages whereas the heterozygous or wild-type larvae grew normally on this diet (Fig. 2A and data not shown).

By contrast, an increase of sucrose content to 10% led to a significant increase in the mutant pupation and especially in the eclosion of adult flies compared with those on a diet containing 5% sucrose (Fig. 2A). The late third-instar larvae were indistinguishable from the wild type on the 10%-sucrose diet; similarly to larvae rescued by the mutation in PhK-γ (Fig. 1C), they did not show any signs of fat body disintegration (image not shown).

In agreement with the suppression screen, the EP779 insertion on PhK-γ, both heterozygous and homozygous, also suppressed the adgf-a mutant phenotype on the 5%-sucrose diet (Fig. 2D). The suppression effect was very weak on a pure yeast diet and only when homozygous insertion was used (Fig. 2D).

The number of circulating hemocytes is greatly increased in the adgf-a mutant and this is associated with a melanotic capsule formation (Dolezal et al., 2005). The sugar-enriched diet decreased the number of circulating hemocytes to a normal level (Fig. 2B) and the mutants very rarely formed melanotic capsules (data not shown). It seems then that the fat body of the adgf-a mutant larvae reared in more restrictive conditions degenerates due to the excessive release of stores, and that the described behavior of hemocytes is a secondary reaction to this degeneration. When the fat body does not degenerate, i.e. on a diet containing 10% sucrose, the hemocytes do not express such behavior.

Sensitivity to dietary restriction is specific for adgf-a

There are five additional ADGF genes with proven or putative adenosine deaminase activity in Drosophila (Zurovec et al., 2002).
Therefore, we decided to test whether the other members of the ADGF family also play a role similar to adgf-a in larval sensitivity to dietary restriction. We used the available combinations of mutants produced by homologous recombination: adgf-c+adgf-d and adgf-a2+adgf-b (Dolezal et al., 2003). Neither of the two combinations showed sensitivity to the dietary restriction (Fig. 2C), demonstrating that none of the four genes (ADGF-A2, ADGF-B, ADGF-C and ADGF-D) played an important role in the observed effects, at least not during larval stages. It is important to stress here that besides ADGF-A, only the ADGF-D gene is expressed during larval development, particularly in the late third-instar larvae, under normal conditions (Zurovec et al., 2002).

We used duplications in the ADGF-A2, ADGF-A, ADGF-B cluster as the adgf-a mutant, particularly lines adgf-aKarel and adgf-aGerda (Dolezal et al., 2003) (see Methods) in which only the ADGF-A gene is mutated on both sides of the duplication. Therefore, we wanted to verify that the observed sensitivity to dietary restriction was solely due to the adgf-a mutation. So, we tested a combination of adgf-aAm2 over adgf-aFr3, which were the true single mutants for the ADGF-A gene recovered by a reduction of the duplications Df(1)Exel9050 (DLR) covering the deficiency Df(1)Exel9050 identified in the genetic screen. The identified deletion Df(1)Exel9050 shown in magenta was recovered from a FLP recombination between P{XP}d00034 (coordinate X:11589014 in the genome release 5.23) and PBac(RB)FucT6e02394 (X:11600657) and covers three genes: PhK-γ, CG2444 and FucT6. Structures of their various transcripts (mRNA) as well as protein coding sequences (CDS) are shown in orange and grey boxes, respectively. The rescue insertion P{XP}PhK-gammaEP779 (EP779) shown in turquoise disrupts one of the three predicted transcripts of the PhK-γ. Corresponding cDNA clones exist for all three variants, verifying that they are all transcribed.
Sensitivity to dietary restriction is suppressed by blocking the signaling through the adenosine receptor

We took advantage of the lethal effects of dietary restriction on the adgf-a larvae to test the role of the extracellular adenosine signaling in these effects. We had previously shown that a mutation in the single Drosophila adenosine receptor (AdoR) suppressed the deleterious effect of elevated levels of adenosine in the adgf-a mutant (Dolezal et al., 2005). As expected, the adoR mutation suppressed the lethality of the adgf-a mutant on the yeast diet supplemented with 5% sucrose, which was similar to cornmeal fly food (Fig. 2D). The same mutation also greatly suppressed the enhanced lethality of adgf-a on the pure yeast diet. Fig. 2D shows that almost all the adoR adgf-a double mutants pupate compared with no pupation of either single adgf-a mutant or mutants homozygous for adgf-a and heterozygous for adoR. A significant number of the double mutants also emerged as adults.
Measurements of the extracellular adenosine and carbohydrates in the hemolymph

The rescue by the mutation in \( \text{PhK} \gamma \) and the sensitivity to dietary restriction indicated that energetic metabolism was affected in the \( \text{adgf-a} \) mutant. Therefore, we measured the levels of circulating carbohydrates in the hemolymph of the control and mutant larvae as one of the markers of energetic metabolism. We collected hemolymph of the pre-wandering third-instar larvae of selected genotypes reared on different diets, eliminated the cells (mainly circulating hemocytes) by centrifugation, and measured the levels of hemolymph glucose and trehalase in parallel with adenosine levels in the same sample using mass spectroscopy analysis.

Similar to our previous observation (Dolezal et al., 2005), although the extracellular adenosine concentration never exceeded 0.2 \( \mu \text{M} \) in the hemolymph of the wild-type or heterozygous \( \text{adgf-a} \) larvae, it increased to 2.5 \( \mu \text{M} \) in the \( \text{adgf-a} \) mutants (Fig. 3A, top). The glucose level in the hemolymph was doubled in the \( \text{adgf-a} \) mutants compared with wild-type larvae (Fig. 3A, middle). The increased survival of the \( \text{adgf-a} \) mutant reared on 10\% sucrose was not associated with a decrease of extracellular adenosine because its level increased similarly with both the 5\%- and 10\%-sucrose diets (Fig. 3A, top). The level of glucose was also similarly increased in the \( \text{adgf-a} \) mutant reared on diets containing either 5\% or 10\% sucrose (Fig. 3A, middle).

The significant increase of circulating glucose in the \( \text{adgf-a} \) mutants is in agreement with the results above, suggesting an affected energetic metabolism. The blocking of adenosine signaling by the \( \text{adoR} \) mutation decreases the level of circulating glucose in the \( \text{adgf-a} \) mutant to a normal level (Fig. 3B, middle), which is in agreement with the suppression of the sensitivity to the dietary restriction. Although the level of extracellular adenosine decreases in the \( \text{adoR adgf-a} \) double mutant compared with \( \text{adgf-a} \), its level is still a magnitude higher than that of the wild type or heterozygous \( \text{adgf-a} \) control (Fig. 3B, top) showing that it is the blockage of AdoR signaling that is responsible for the observed rescue effects. Interestingly, the extracellular adenosine is similarly increased in the \( \text{EP779; adgf-a} \) double mutant and in \( \text{adoR adgf-a} \) (Fig. 3B, top) but, compared with \( \text{adoR} \), the mutation in \( \text{PhK} \gamma \) does not lower the glucose level in the hemolymph of the \( \text{adgf-a} \) mutant (Fig. 3B, middle).

We also measured trehalose, a non-reducing sugar formed from two glucose units, which is present both in circulation and tissues and serves as energy storage (glucose can be rapidly produced by trehalase) and protectant molecule in various stresses in prokaryotes, yeasts, plants and invertebrates (Elbein et al., 2003). Trehalase and serves as energy storage (glucose can be rapidly produced by trehalase).

We collected the \( \text{adgf-a} \) mutant larvae and their heterozygous siblings reared on cornmeal diet at different times to produce a developmental profile of the hemolymph metabolites in the third-instar larvae heterozygous for the \( \text{adgf-a} \) mutation. Three time-points are shown: 90- and 102-hour-old larva (still feeding) and non-feeding wandering larva in which the glucose level decreases and trehalose increases. The x-axis represents concentration of adenosine (top panels), glucose (middle panels) and trehalose (bottom panels) in mmol/ml (\( \mu \text{M} \)); data are represented as mean values ± s.e.m. The x-axis labeling is the same for all graphs in each panel.

Measurement of carbohydrate stores in larvae

We collected the \( \text{adgf-a} \) mutant larvae and their heterozygous siblings reared on cornmeal diet at different times to produce a developmental profile of carbohydrate stores, i.e. glycogen and trehalose. Fig. 4A shows that normal larvae progressively accumulated glycogen stores while feeding, reaching a peak before they start wandering. Then after the larvae stop feeding, which was associated with a decrease in hemolymph glucose (Fig. 3C, middle), the glycogen stores started a rapid decline. At least part of the glycogen utilization probably goes into the production of trehalose, the concentration of which increased in the hemolymph during wandering (Fig. 3C, bottom).
Fig. 4A demonstrates that the \textit{adgf-a} mutant larvae accumulated glycogen stores more slowly than heterozygous siblings and that they were not able to reach the pre-wandering peak present in the heterozygous siblings. There was a similar amount of glycogen stores in the prepupal stage of those \textit{adgf-a} mutants reaching this stage as in heterozygous siblings. Similarly to glycogen, the total trehalose also accumulated slower in the \textit{adgf-a} mutant than in normal larvae but eventually similar levels were reached.

We also tested an effect of fasting on the carbohydrate stores. Fig. 4B shows that the speed of the glycogen breakdown was similar...
in the \textit{adgf-a} and normal larvae, i.e. after 4 hours half of the stores were utilized. Interestingly, the \textit{EP779} mutation in \textit{PhK-\(\gamma\)} \textit{sl} slowed down the glycogen breakdown in the \textit{adgf-a} mutant (Fig. 4B). Thus, the slower glycogen breakdown achieved by the \textit{EP779} insertion might help the \textit{adgf-a} larvae to accumulate more glycogen stores, which is in agreement with the rescue effect found in the genetic screen. Although there were not such dramatic changes in trehalose levels during starvation (larvae seem to preferentially use glycogen stores), \textit{EP779} insertion also resulted in a different speed from normal and \textit{adgf-a} larvae.

**Extracellular adenosine stimulates energy release from the fat body in parallel to AKH**

Because an adipokinetic hormone (AKH) stimulates the release of energy stores in Drosophila (Lee and Park, 2004), we decided to test whether the glucose release observed in the \textit{adgf-a} mutant was due to the action of AKH. Using the UAS/Gal4 system (Brand and Perrimon, 1993), the AKH producing cells in corpora cardiaca were ablated by expressing the pro-apoptotic gene \textit{Grim} (UAS-\textit{Grim}) (Zhou et al., 1997) driven by the AKH-Gal4 driver (Isabel et al., 2005) which is expressed exclusively in the AKH-producing cells in the corpora cardiaca (Fig. 5C). The complete ablation of these cells in otherwise wild-type larvae was vital (Isabel et al., 2005) (Fig. 5A). However, it did not lead to the increased survival of \textit{adgf-a} mutant larvae (there was a small increase in number of adult flies on cornmeal and 5%-sucrose diet). The ablation of AKH-producing cells in the \textit{adgf-a} mutants (Fig. 4C) resembled the \textit{adgf-a} phenotype for all types of diets (Fig. 4A,B), demonstrating that AKH was not significantly involved in the observed effects of adenosine on the energy metabolism of larvae.

AdoR, similarly to AKH receptors, is coupled to G proteins. Although AdoR expression was previously observed using in situ hybridization in the ring gland where AKH-producing cells are also located, it was not detected in the fat body (Dolezelova et al., 2007). However, the AdoR expression was very weak, producing faint signals. Therefore, we used the more sensitive RT-PCR method to check the expression in fat body tissue isolated from the third-instar larvae. Fig. 5D demonstrates that the AdoR was indeed expressed

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**Fig. 5. Ablation of AKH-producing cells and analysis of AdoR expression.** (A,B) Effect of an AKH-producing cell ablation on the viability of the \textit{adgf-a} mutant. The ablation by activation of proapoptotic gene \textit{Grim} driven by AKH-Gal4 does not affect development of otherwise wild-type larvae when compared with either \textit{yw} or heterozygous \textit{adgf-a} controls on cornmeal fly food (A). AKH-Gal4 alone in the \textit{adgf-a} mutant resembles the \textit{adgf-a} mutant phenotype and activation of \textit{Grim} by this AKH-Gal4 shows no effect on \textit{adgf-a} larval survival (\(P=0.23\)) and a slight effect on the number of adult flies (\(P=0.028\)) (A). Similar results are achieved using a pure yeast diet (pupae \(P=0.36\), adults \(P=0.25\)) and yeast diet supplemented with 5% sucrose (pupae \(P=0.35\), adults \(P=0.01\)) (B). Data are represented as mean percentage ± s.e.m. (C) Immunohistochemical analysis of AKH-producing cells. AKH is produced and kept ready for secretion in a cluster of ring-gland cells called the corpora cardiaca (CC) both in wild-type and \textit{adgf-a} mutant larvae, as demonstrated by staining with anti-AKH antibody (red). These cells are completely ablated by the activation of proapoptotic gene \textit{Grim} by AKH-Gal4, demonstrated by missing signal in the ring gland (depicted by white dots) of the \textit{adgf-a} mutant. Nuclear staining by DAPI (top panels) as well as DIC (bottom panels) is shown for morphological visualization. Dissected wild-type ring gland is shown in higher magnification (CA corpora allata; PG prothoracic gland); \textit{adgf-a} ring gland is shown attached to the brain. (D) Gel image showing RT-PCR of AdoR. RNA was extracted from fat bodies of \textit{yellow white} (\textit{yw}) and \textit{Oregon-R} (\textit{Or-R}) larvae. Both fat bodies (\textit{yw} fat body and \textit{Or-R} fat body) and the remaining tissue after the fat body dissection (\textit{yw} and \textit{Or-R}) were analyzed. Control without reverse transcriptase (RT–) is also shown to demonstrate that the PCR product originates from reversely transcribed RNA and not from a genomic DNA contamination. Actin serves as a control for the template amount.

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in this tissue. This suggests that it is direct AdoR signaling to the fat body, working parallel to AKH signaling and using the same downstream machinery of G proteins, that is responsible for the observed effects of adenosine.

**DISCUSSION**

**Extracellular adenosine increases a circulating glucose**

We previously showed that a deregulation of the adenosine levels due to the deficiency of Drosophila ADGF-A leads to larval or pupal death associated with a degeneration of larval fat body (Dolezal et al., 2005). We used a genetic screen, which led us to the identification of a mutant in the γ-subunit of the phosphorylase kinase (PhK-γ) suppressing the adgf-a mutant phenotype. PhK activates through the phosphorylation of glycogen phosphorylase (glycogenolysis) (Fig. 6). Thus, the suppressive effect of PhK-γ mutation corresponds to the observation that the fat body, the main organ for glycogen storage, does not degenerate in the rescued larvae. This rescue led us to a recognition that carbohydrate metabolism might be affected in the adgf-a mutant larvae.

The striking sensitivity of the adgf-a mutant to dietary restriction strongly supports this conclusion. We used a pure yeast diet, which is rich in proteins but poor in carbohydrates (Buch et al., 2008). The use of this diet led to enhanced larval lethality of the adgf-a mutant, whereas the wild-type larvae developed normally on this food. Supplementing the pure yeast diet with 5% sucrose gave larvae resembling the adgf-a mutant phenotype on the cornmeal-based fly food. An increase of sucrose to 10% then leads to a significantly higher mutant survival rate, with a greatly improved morphology of the mutant larvae and most of them not showing any sign of fat body degeneration. Thus, although lowering carbohydrates enhances the mutant phenotype, increasing the amount of carbohydrate in the diet most probably compensates the loss of energy reserves or allows the saving of more stores than in the restrictive conditions. Saving stores of energy is crucial for the larvae to continue in their development. The survival of the adgf-a mutant larvae is thus critically dependent on the amount of carbohydrate in the diet.

Finally, an analysis of carbohydrate concentrations in the hemolymph demonstrated that the balance between energy storage and release was indeed shifted in the adgf-a mutant towards release because the mutant larvae presented doubled levels of glucose in the hemolymph compared with the wild type. The elevated levels of extracellular adenosine in the adgf-a mutant are thus associated with hemolymph hyperglycemia.

Extracellular adenosine was not lowered in the mutant larvae rescued on a 10%-sucrose diet compared with mutants on a 5%-sucrose diet, demonstrating that the rescue by increased sugar is not due to lowering of the extracellular adenosine level. The level of glucose in the hemolymph was also similarly increased, most probably because the elevated adenosine increases the circulating glucose to these levels. Therefore, it is plausible that increasing the amount of carbohydrates in the diet allows the saving of more stores, while still keeping the hemolymph glucose levels high. This in turn is expressed as a thick fat body of the rescued larvae on 10% sucrose. The rescue by diets higher in sucrose further demonstrates that it is not hyperglycemia (at this level) that kills the adgf-a mutants.

The measurement of carbohydrate stores demonstrated that the accumulation of both glycogen and trehalose during feeding period is much slower in the adgf-a mutant larvae than in heterozygous siblings most probably because the elevated adenosine forces the circulating glucose levels to be kept high. This might have fatal consequences for larvae on a diet poor in carbohydrates that does not allow larvae to save enough stores to proceed normally in development, as seen with the adgf-a mutant on a pure yeast diet. Slowing down the glycogen breakdown (by mutation in PhK-γ, as seen in Fig. 4B) does not help much in this situation; EP779 increased the survival of adgf-a poorly on a pure yeast diet. However, it does help larvae on a 5%-sucrose diet, which seems to provide adgf-a with just the minimum amount of carbohydrate, sufficient to save enough stores, at least for some larvae (about a third of them) to proceed in development. Probably any situations requiring a mobilization of the energy from the glycogen stores shifts the thin balance on 5% sucrose so it does not allow the adgf-a mutants to pupate. In these conditions, slowing down the glycogen breakdown helps the adgf-a mutants, as demonstrated by a significant increase in pupariation rate of the EP779; adgf-a mutants on 5% sucrose.

**Adenosine via AdoR functions as an anti-insulin hormone in parallel to AKH**

The main storage organ in insects is the fat body, a counterpart of mammalian liver and adipose tissue. The deposition of stores as well as the stimulation of their release is intensively studied in many insect models, which show that the basic regulation of carbohydrate metabolism is similar to that in higher organisms (Hudson et al., 1993). As shown in Fig. 6, glycogen synthesis and breakdown is
regulated by glycogen synthase and glycogen phosphorylase. The energy-demanding processes in insects stimulate the release of energy from the fat body stores through the action of AKHs (reviewed in Gäde and Auerswald, 2003). AKH, a counterpart of mammalian glucagon, binds to G-protein-coupled receptors and, in the case of carbohydrate metabolism, activates glycogen phosphorylase via cAMP production and Ca^{2+} (binding to G_{i} and G_{q} subunits). This includes regulation by Ca^{2+}-dependent PhK.

Extracellular adenosine can affect carbohydrate metabolism in the fat body by two possible mechanisms (Fig. 6): signaling through AdoR, and intracellularly by transport via adenosine transporters (Thorn and Jarvis, 1996) and a conversion to AMP by adenosine kinase inside the cell (Van De Wiele et al., 2002). Glycogen phosphorylase is, in addition to phosphorylation by PhK, also activated by allosteric effectors including AMP (Tsitsanou et al., 1999). AMP can also inhibit glycogen synthase through the action of AMP-activated protein kinase (AMPK) (Towler and Hardie, 2007). However, we show here that the blockage of AdoR signaling almost completely rescues the mutant larvae on a pure yeast diet. This is supported by resetting the glucose level in the hemolymph of the adoR adgf-a double mutants back to normal levels. These results clearly demonstrate the role of AdoR signaling in the regulation of carbohydrate metabolism in Drosophila. A similar role for adenosine signaling through adenosine receptors stimulating the glucose release was described for mammalian liver cells (Gonzalez-Benitez et al., 2002), suggesting that this role of extracellular adenosine and the mechanism of action are evolutionary conserved from flies to mammals.

Because AdoR is expressed in the Drosophila ring gland (Dolezelova et al., 2007) where the AKH-producing cells are located (Lee and Park, 2004; Isabel et al., 2005), we speculated that the effect of adenosine could be triggered by AKH release in response to AdoR signaling in the ring gland. However, the ablation of AKH-producing cells did not rescue the adoR mutant whereas the adgf-a sensitivity of the adgf-a mutation does. The extracellular adenosine thus instructs the organism to keep hemolymph glucose (originating most probably from dietary sucrose) levels high, while lowering glycogen storage. Therefore, there might be an additional AdoR signaling effect, besides that on glycogenolysis, that influences how much of the carbohydrate obtained from food will be used for storage and how much will be kept in the hemolymph for immediate use (Fig. 6).

Extracellular adenosine can be then seen as a true anti-insulin hormone. This agrees with a concept proposed by the most recent work of Piñá’s group, that adenosine can play an even stronger anti-insulin hormone-like role in the mammalian system under certain conditions than classical anti-insulin hormones such as adrenaline or glucagon (Cortés et al., 2009). It is important to stress that although our study focuses solely on carbohydrate metabolism, adenosine might have a similar effect on lipid metabolism.

**Extracellular adenosine: a candidate signal involved in wasting**

The observation that accumulated extracellular adenosine can lead to death of the organism through an impairment to the process of saving energy stores might help to better understand certain pathologies. The immune response is energetically costly (Derting and Compton, 2003; Freitak et al., 2003) and a progressive loss of energy stores – also called wasting – is observed during certain chronic infections in both flies and humans (Dionne et al., 2006; Lazzaro and Galac, 2006). In spite of a potentially high biomedical importance, the network of the molecular signals leading to wasting is largely unexplored. Results of this work suggest that extracellular adenosine could be one of those signals.

Adenosine is well known as a stress hormone and it is produced during the immune response (Hasko et al., 2002); therefore it could play an important hormonal role in energy allocation during stress conditions such as infection. Our previous work suggests a connection between adenosine signaling and Toll signaling, which is an important regulator of the immune response in flies (Dolezal et al., 2005). A requirement to use virtually sterile conditions to obtain reproducible results in this work (see Methods) suggests a high sensitivity of the adgf-a mutants to infectious stress. Microarray analysis in the fly model for wasting also shows changes in the expression of the adenosine-regulating enzymes (Dionne et al., 2006). It is important to further investigate this intriguing role for extracellular adenosine, and our work presented here demonstrates that Drosophila can be now utilized as a useful, genetically tractable model to investigate the stress-hormone-like roles of extracellular adenosine in energy allocation in different conditions in vivo.

**Concluding remarks**

We have shown that (1) increased extracellular adenosine due to adenosine deaminase deficiency leads to high levels of glucose (hyperglycemia) in the hemolymph during Drosophila larval development. (2) This effect is carried out by adenosine signaling through the adenosine receptor. (3) Adenosine works in this regard as an anti-insulin hormone in parallel to AKH, a counterpart of mammalian glucagon. This role and mechanism seems to be well conserved between flies and mammals, making Drosophila a good model for these effects. (4) The hyperglycemia associated with the extensive adenosine signaling further leads to the impairment in building carbohydrate stores during the larval period and to high sensitivity to sugar restrictions in the diet. This can be partially suppressed by lowering the glycogen breakdown by a mutation in PhK. In suboptimal conditions or with diet restrictions, the described effects can lead to death of the organism. Because adenosine signaling is associated with the immune response and the response to stress in general, our results make extracellular...
adeno sine a good candidate for a signal involved in the wasting syndrome, which has an impact on various pathologies.

METHODS
Fly strains
The adgf-a mutant (chr. III) was prepared by crossing w; adgf-a\(^{Karel}\)/TM3 Ser Act-GFP with w; adgf-a\(^{Gerda}\)/TM3 Ser Act-GFP and selection was based on the lack of GFP in w; adgf-a\(^{Karel}\}/adgf-a\(^{Gerda}\) larvae. Both parent lines carried duplications of the ADGF-A2, ADGF-A, ADGF-B cluster interrupted by a mini-white marker in which only the ADGF-A gene was mutated on both sides of each duplication (Dolezal et al., 2003). Homozygotes for each duplication as well as transheterozygotes adgf-a\(^{Karel}\}/adgf-a\(^{Gerda}\) were functionally verified to be a loss-of-function mutant just for the adgf-a gene (Dolezal et al., 2003). Because these two lines were produced independently and always kept separately, their combination was used as the adgf-a mutant to avoid the effects of any side mutations. This combination also gave us the advantage of the mini-white marker presence, making certain crosses easier. Additional lines that were mutant for the ADGF-A gene without the mini-white marker (adgf-a\(^{Am2}\) and adgf-a\(^{Pr}\)) (Dolezal et al., 2003) were used only when specified in the text. Other strains used in this work included: adgf-a2 adgf-b and adgf-c adgf-d (double mutants on chr. III) (Dolezal et al., 2003); adOR (mutant in the adenosine receptor on chr. III) (Dolezal et al., 2005); adOR adgf-a\(^{Karel}\) and adOR adgf-a\(^{Gerda}\) recombinant double mutants; P(EP)PhKgamma[EP779] (on chr. X, BDGP disruption project); UAS-Grim (insertion on chr. II, gift of John Nambu, University of Massachusetts, Amherst, MA); AKH-Gal4 (pAKH02 insertion on chr. III, gift of Jan Veenstra, CNRS, University of Bordeaux, France).

Genetic screen for the suppressors of the adgf-a phenotype
A Bloomington deficiency kit for chromosome X and II (a set available in 2005, generated by the Bloomington Deletion Project, the DrosDel Project and Exelixis) was used for the screen. Deletions were crossed with the adgf-a mutation as shown in supplementary material Fig. S1. The adgf-a homozygous progeny were scored for numbers of pupae and adult flies and for the presence of melanotic capsules, in three consecutive vials.

Feeding and developmental assays
Flies were grown on standard cornmeal medium (8% cornmeal, 5% sugar, 4% yeast, 1% agar, 0.16% methylparaben). To count viability and observe the development, flies were allowed to lay eggs for 4 hours on agar juice plates supplemented with fresh yeast paste. Embryos of ~20 hours old were collected by brush, washed in water to clean the yeast, and washed for 30 seconds in 96% ethanol to eliminate infectious agents. The ethanol-treated embryos were transferred to sterile agar plates with the desired diet to complete embryonic development. The early feeding between hatching to the first-instar larvae and the transfer to vials seemed to have an impact on the selection of feeding experiments; therefore, the washed embryos were always transferred to the same diet as was in the vial. The first-instar larvae of desired genotype scored by GFP expression were collected with a sterile needle into vials (20-30 individuals per vial) supplemented with 80 μl of penicillin/streptomycin solution (Sigma; 10000 U penicillin/10 mg streptomycin per ml). The washing of embryos and treatment with penicillin/streptomycin appeared to be crucial for obtaining consistent data because the adgf-a mutant seems to be very sensitive to any environmental stress. The development from larva to adults was observed and scored for each individual vial. All counting experiments were done at least in triplicates and repeated at least twice at different times.

Yeast-based diets
For the dietary restriction experiments, the diets were prepared as follows: dry yeast and agar were dissolved in distilled water to achieve 8 and 1.2% w/v, respectively. The solution was boiled for 10-15 minutes to inactivate live yeast while maintaining the total volume. At the end, sucrose was added to make 5 or 10% w/v (for the pure yeast diet, no sucrose was added) and briefly boiled. The suspension was cooled down to ~60°C, methylparaben (Sigma) was added to 0.16% and the final diet was poured into the vials. A few hours before collecting the first-instar larvae, 80 μl of penicillin/streptomycin solution was added on the surface of each vial and allowed to dry. Agar plates for washed embryos contained the same diet with 4% agar.

During experiments we noticed that the length of cooking is important because cooking for more than 30 minutes led to a decrease in the adgf-a mutant survival (not the wild-type control). It seems that certain carbohydrate constituents (maybe also other important nutrients) present in the yeast and/or cornmeal become unavailable with longer cooking or boiling in a microwave oven. Therefore, we used strictly 30 minutes at 90°C using a thermostirer and all comparing experiments were done using a diet from the same cooking batch.

Depletion of AKH-producing cells
We crossed flies carrying UAS-Grim with flies carrying AKH-Gal4 to ablate AKH-producing cells. In the rescue experiments, AKH-Gal4 was recombined with chromosome carrying adgf-a\(^{Karel}\) to obtain UAS-Grim/++; AKH-Gal4 adgf-a\(^{Karel}\}/adgf-a\(^{Gerda}\). The presence or absence of AKH-producing cells was determined by wholemount immunostaining of the third-instar larvae using a primary rabbit anti-AKH antibody (Isabel et al., 2005), secondary anti-rabbit Cy3.5 (Rockland) and DAPI staining.

RNA extraction and RT-PCR
Oregon-R and yw larvae of the late third-instar (118 hours after egg laying) were dissected in Ringer solution and the fat body separated from the rest of the larval body. Total RNA was then isolated from both fat body tissues and the remaining carcass using Tri Reagent (MRC), following the manufacturer’s instructions. Isolated RNA was treated with Turbo DNase (Ambion) and reversely transcribed into the first strand cDNA using Superscript III (Invitrogen). Control reactions (RT-) were prepared simultaneously without the addition of Superscript. The following primers were used for PCR amplification of Actin: Actin5SC 5’-TACCCCATTTGACCGGTAT-3’ and Actin5CR 5’-GGTCATCTTCTACGGTTG-3’; for amplification of AdoR: Probe2F 5’-AGATCTCGAGCGAAGAATGTTGGAAC-3’ and Probe2R 5’-CTCTGAGGCTGGCACCATTG-3’. AdoR was amplified by 34 cycles without the additional final elongation step.
Measurement of hemolymph metabolites

Hemolymph was collected from third-instar pre-wandering larvae. Some 10–15 larvae were transferred onto a paraffilm-covered microscopic slide on ice in 1 μl of inhibition solution (1 mM thiourea, 20 mM EDTA). Then, 3 μl of the hemolymph were transferred by automatic pipette into 60 μl of cooled extraction medium [MeCN/water 8:2 (v/v) with 20 mM ammonium acetate, pH 7.5–8.1, spiked with the 13C-labeled standards: 0.5 μM 13C5-adenosine and 20 μM 13C6-glucose], mixed and centrifuged (1600 g for 2 minutes at 4°C). To 50 μl of the supernatant, 50 μl of MeCN was added and the mixture stored at −20°C until analysis using liquid chromatography coupled with tandem mass spectrometry. HPLC analysis was carried out on a quaternary Rheos Allegro UPLC system (Thermo, San Jose, CA) equipped with an HTC PAL autosampler system (CTC Analytics, Switzerland) cooled at 5°C during analysis. Separation of the analytes was performed on a Luna hydrophilic interaction liquid chromatography column (150 mm × 2.0 mm × 3 μm; Phenomenex, Torrance, CA) at room temperature with an injection volume of 5 μl. Gradient elution at a flow rate of 250 μl/minute was used, with solvent A [MeCN/water 95:5 (v/v) with 20 mM ammonium acetate, pH 8.1] and solvent B [MeCN/water 5:95 (v/v) with 20 mM ammonium acetate, pH 8.1] going from 90:10 (A/B) to 20:80 (A/B) in 10 minutes, followed by equilibration at 90:10 (A/B) for 8 minutes. The detection was carried out using a LTQ XL linear ion trap mass spectrometer (Thermo) equipped with an electrospray ion source operated simultaneously in the positive and negative ion mode, setting the sprayer voltages at 4.5 and –3.5 kV, respectively, and the ion source temperature at 350°C. Positive ions were monitored in the selected ion monitoring (SIM) scan mode by monitoring the transitions m/z 368.2 and 373.2 respectively, and the ion source temperature at 350°C. Positive ions were obtained by the standard addition method, by means of the previously measured hemolymph samples spiked with known concentrations of native standards against 13C5-adenosine (i.e. adenosine) and 13C6-glucose (glucose and trehalose).

Measurement of carbohydrate stores

Larvae of desired age were washed from the diet with cold PBS. Some10-15 larvae were crushed in 200 μl of PBS on ice, then immediately heated to 70°C for 7 minutes. Before heating, an aliquot for the protein measurement was taken. Samples were stored at –20°C (protein aliquots at –70°C). The thawed samples were centrifuged at 15,000 g for 7 minutes. The supernatant was transferred to a new tube. Glucose was measured with the kit GAGO-20 (Sigma) in 96-well plates. Enzyme solution (100 μl) was added to 45 μl of the sample and incubated for 30 minutes at 37°C. The reaction was stopped with 100 μl of 12 N H2SO4 and measured at 540 nm within 10 minutes of stopping the reaction. Glycogen and trehalose were first converted to glucose with amyloglucosidase (Fluka-Sigma 10115) or trehalase (Sigma T8778) in 30 μl of reaction mixture per well and measured with the kit GAGO-20. For glycogen, 5 μl of 100 U/ml solution of amyloglucosidase was added to 4 μl of sample and filled to 30 μl with PBS. Then, 100 μl of GAGO-20 enzyme solution were added and the reaction incubated at 37°C for 40–50 minutes. For trehalose, 3 μl of the sample was mixed with 2 μl PBS and 25 μl trehalase buffer (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl and 14.8 mM/ml trehalase) (Teleman et al., 2005), incubated for 20 hours at 37°C, and then measured with GAGO-20. Proteins were measured by the Bradford assay.

For starvation experiments, larvae were collected at 108 hours after laying eggs and were starved for either 2 or 4 hours on filter paper moistened with PBS.

Circulating hemocytes

Circulating hemocytes were counted as previously described (Dolezal et al., 2005).

**TRANSLATIONAL IMPACT**

**Clinical issue**

Breakdown of excessive amounts of adenosine triphosphate (ATP), the main energy source of a cell, leads to the formation of extracellular adenosine, an important signaling molecule involved in multiple pathways that regulate neuronal and immunological function. Adenosine is either actively produced by certain cells or is released from damaged tissue, particularly when cells are starved of oxygen. Adenosine levels are tightly regulated at all points throughout its production, release, uptake and degradation, and disruptions in control that result in excessively high levels are associated with a number of human pathologies, including severe combined immunodeficiency (SCID). The diversity in the regulation and function of adenosine (both of which differ in a tissue- and cell-specific manner) means that it has been difficult to unravel its exact role at the molecular level. In addition, data obtained in vitro, or in tissue culture, might not be relevant to whole organisms, and mammalian model systems are overly complicated. It would therefore be very useful to have a simpler model.

**Results**

The authors previously showed that, in Drosophila, increasing extracellular adenosine by knocking out the adenosine deaminase gene (adaf-g) is fatal. Here, they demonstrate that larvae with increased adenosine are hyperglycemic, and cannot accumulate energy stores in the form of glycogen for their further development; in diet-restrictive conditions, these effects lead to death of the fly. Death can be prevented by mutating AdoR, the Drosophila adenosine receptor gene, which shows that the signaling function of adenosine is required for the lethal phenotype. The disruption of glucose metabolism is probably due to adenosine directly acting to stimulate glucose release in parallel with adipokinetic hormone, the Drosophila counterpart of glucagon.

**Implications and future directions**

The identification of extracellular adenosine as a stimulator of glucose release in Drosophila supports a recent study in rats suggesting that stress-induced release of extracellular adenosine has a strong anti-insulin effect. The machinery of adenosine signaling seems to be well conserved between flies and mammals, making Drosophila a potentially useful model in which to study the effects of extracellular adenosine on energy metabolism. The link between excess adenosine and glucose metabolism might be of clinical importance because there is frequently a harmful early-phase hyperglycemia in severely traumatized patients. A second intriguing observation concerns the inability of adgf-a mutants to store glycogen; if this effect is conserved in mammals, it could be important in the molecularly unexplored phenomenon of wasting, which is caused by a progressive loss of energy reserves during certain infections.

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Statistical analysis

The following statistical tools were used: two-sample assuming equal variances t-test, ANOVA Tukey’s HSD (honestly significant difference) test pro, and multiple comparison among regressions Tukey’s HSD test (Zar, 1996).

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COMPETING INTERESTS

The authors declare no financial and competing interests.

AUTHOR CONTRIBUTIONS

M.Z. and T.D. conceived and designed the experiments. M.Z. performed most of the experiments, M.F. performed experiments using AKH, and T.D. with help of L.J. performed the original genetic screen. M.Z., M.F., P.S. and T.D. analyzed the data. P.S. contributed mass spectrometry analysis tools. M.Z. and T.D. wrote the manuscript with M.F. providing useful comments.

SUPPLEMENTARY MATERIAL

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Increased adenosine leads to wasting


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