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Adenine nucleotide metabolism during anoxia and postanoxic recovery in insects

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Abstract. Severe hypoxia (anoxia), if maintained for more than a few minutes, causes irreversible damage in humans and other mammals. Why mammals are so vulnerable to anoxia is not fully understood. It is therefore of interest to study animals that are more tolerant of anoxia in order to identify physiological and metabolic properties that are correlated with a high tolerance of anoxia. Insects have high metabolic rates and their energy metabolism is dependent on aerobic ATP production. In insects, as in mammals, anoxia causes a rapid breakdown of physiological function, resulting in a state similar to rigor mortis. This is accompanied by a precipitous decrease in metabolic rate. In contrast to mammals, however, insects can survive anoxia for many hours and recover spontaneously and completely when air is again available. We have followed the metabolism of adenine nucleotides in locust tissues (mainly in the flight muscle) over 3 h of anoxia and during recovery from 1 h of anoxia. The content of ATP in the flight muscle dropped to 1% of normal during 2 h of anoxia. The main product was AMP which increased in content more than 20-fold. Some of the AMP was deaminated to IMP and this was further dephosphorylated to inosine. Altogether less than 30% of the total adenine nucleotides were degraded during 3 h of anoxia and this may contribute to the amazing ability of insects to recover from prolonged anoxia.
Key words. Hypoxia/anoxia; insects; mammals; metabolism of adenine nucleotides; tissue damage; oxygen-free radicals.

Introduction

Tissue hypoxia, the insufficient supply of oxygen to tissues, is a common and serious clinical problem. It is the most frequent cause of death in humans. Some vital organs, particularly brain and heart, are very vulnerable to hypoxia. Humans share this sensitivity with other mammals and with birds[28, 30, 31]. Why higher vertebrates are so sensitive to hypoxia is not fully understood. However, it is commonly held that some properties of their energy metabolism, such as a high basal metabolic rate and the dependence of vital organs on aerobic ATP production, are incompatible with a high tolerance of hypoxia, i.e. the ability to survive extended intervals of insufficient oxygen supply. High metabolic rates and dependence on aerobic ATP production are also evident in adult insects, yet insects have been found to be much more tolerant to hypoxia than mammals[29]. Humans cannot fully recover if deprived of oxygen for more than 5 to 8 min (the interval is even shorter in small mammals or birds) whereas many insects survive, with no obvious damage, for several hours in an atmosphere devoid of oxygen (strict hypoxia, anoxia). Insects are therefore interesting experimental animals for the identification of those properties of tissue structure and metabolism that are connected with a high tolerance of hypoxia/anoxia.

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The main problems during hypoxia originate from the lack of biological energy in the form of ATP. In both insects and higher vertebrates (as in the majority of other animals) almost all the ATP is produced by oxidative phosphorylation. If oxygen is lacking, ATP synthesis will be insufficient. As a consequence, more ATP will be degraded than is regenerated. Although products of ATP catabolism can be lost from the cells, this loss is not the only problem that could arise from adenine nucleotide catabolism during hypoxia. Evidence has accumulated that part of the anoxic damage is actually initiated not during but after an anoxic (ischaemic) interval when oxygen is again available. Products of ATP breakdown can react with oxygen and thus generate very reactive and hence highly toxic oxygen species such as hydrogen peroxide H_2O_2, superoxide anion $O_2^{-\cdot}$ and the hydroxyl radical $OH^{\cdot}$.

In this brief review we will focus on experiments with locusts, and we will discuss these in the context of how the tolerance of anoxia is related to (a) basal metabolic rate and its depression during anoxia, (b) metabolism of adenine nucleotides during hypoxia and post-hypoxic recovery, and (c) the hypoxia-induced formation of reactive oxygen species.

Insect metabolism under normoxic conditions and acute effects of hypoxia/anoxia

In insects oxygen is transported to the tissues not by means of chemical carriers but in gaseous form via the

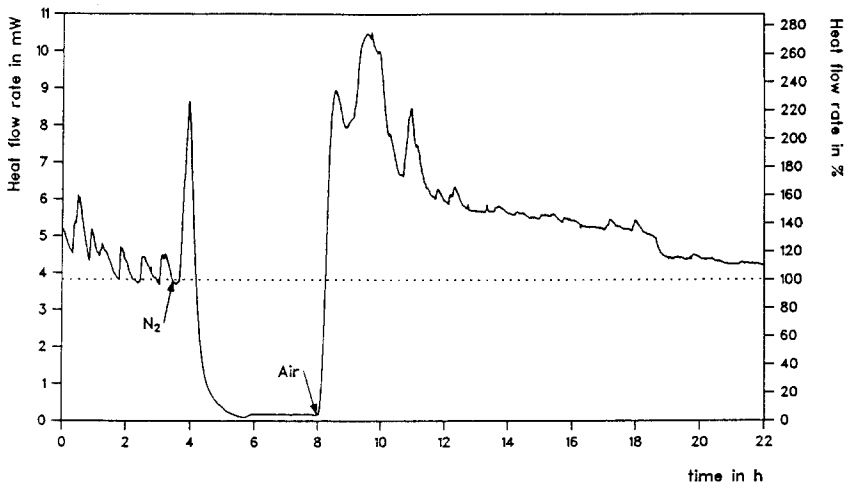


Figure 1. Effects of hypoxia/anoxia and post-anoxic recovery on the heat flow rate (metabolic rate) of an adult locust. A male locust was placed in a microcalorimeter cell through which a flow of artificial air was passed at a constant rate of 500 ml/h at 26.4 °C. Anoxia was produced by switching the flow from air to pure nitrogen. Heat flow rate was initially unchanged but when hypoxia became critical ($<2\%$ O_2) a transient hyperactivity caused a distinct peak of heat flow which was followed by a precipitous decrease in heat flow rate to less than 5% of the normoxic value. When air was available again, heat flow rate increased rapidly to more than twice its normoxic value and remained elevated for some time before slowly returning to its normoxic level[32].

tracheal system. The tracheae form an intricate system of tubules, the finest ramifications of which (tracheoles) can enter cells to end in close proximity to mitochondria[34]. Insects thus take advantage of the high oxygen content of air and the much faster diffusion of O_2 and CO_2 in air as compared with fluids[28]. Insects therefore do not normally experience lack of oxygen in their natural habitats, their metabolic rates are high and ATP synthesis is based on aerobic processes.

Like mammals insects are not able to function anaerobically, even for very short intervals. The acute effects of anoxia on insects are dramatic. A locust, for instance, when exposed to an atmosphere of pure nitrogen will immediately give up its resting position. A brief period of escape movements will be followed by rapid loss of physiological functions when the locust loses control of its body posture and falls onto its side. Violent twitching of the hind legs and trembling of the extremities are followed by complete immobility within about two minutes. Like insects, mammals show dramatic reactions if subjected to anoxia (or systemic ischaemia): rapid loss of consciousness, followed by anoxic seizures and complete immobility[7,31]. In both cases the acute response to anoxia gives evidence that a highly active central nervous system (CNS) cannot function if aerobic ATP production is halted (see below). Some lower vertebrates, however, are able to delay a breakdown of the nervous system during anoxia by reducing neuronal function of the CNS. Frogs, for instance, which are moderately tolerant of anoxia, show a gradual metabolic depression of parts of the CNS during severe hypoxia[30]. Turtles and crucian carp can survive long periods of anoxia; hypoxia was shown to trigger the release of inhibitory neurotransmitters within the brain, and this has been proposed as part of a mechanism to stabilise brain homoeostasis and to delay anoxic tissue damage[14,20].

Metabolic rate during hypoxia and posthypoxic recovery

If oxygen is a limiting factor, metabolic rates cannot be determined by measuring the gas exchange (O_2 uptake and/or CO_2 production, indirect calorimetry). Instead heat dissipation (direct calorimetry) can be used for estimating metabolic rates. Microcalorimetry has been employed to study insects during hypoxia, anoxia and post-anoxic recovery[18,32]. The time course of heat flow rate is very characteristic (see fig. 1). When the gas flow through the calorimeter is changed from air to pure nitrogen a distinct peak of heat flow is observed, which reflects the brief hypoxia-induced hyperactivity of the insect. This transient increase in heat flow rate is followed by a precipitous decrease to less than 5% of the normoxic rate. If air is again supplied, heat flow is rapidly increased to twice or more the normoxic control value and returns very slowly to this value (fig. 1).

Unlike most adult insects which have a constant supply of oxygen, some animals regularly encounter hypoxic intervals in their natural habitats. For example, species living in the tidal zone of the sea cannot take up oxygen from the water during low tide, and lower vertebrates in fresh water may be cut off from air when ice forms at the surface of the water during winter. These animals have evolved adaptations to cope with transient environmental hypoxia/anoxia. Most important is the reduction in metabolic rate during anoxia; heat flow rates have been reported to decrease to between 15% and

30% of the respective normoxic rates. However, the physiological and metabolic processes that go along with the hypoxic depression of metabolism are different in animals adapted to hypoxia, and in adult insects that do not normally experience lack of oxygen. This is most striking with respect to ATP and its catabolites, as will be discussed in the next section.

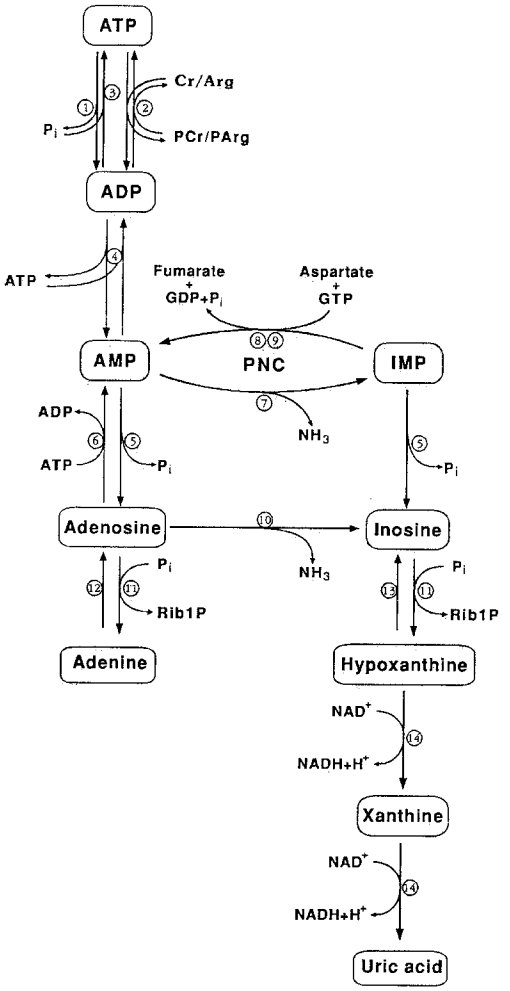
Metabolism of adenine nucleotides during anoxia

Almost all physiological processes in animals are driven by hydrolysis of ATP. The capacity for work depends on the phosphorylation potential ($[ATP]/([ADP] \cdot [P_i])$) which must hence be kept sufficiently high. This is achieved by the constant regeneration of ATP by the reaction ADP + inorganic phosphate → ATP.

In a typical animal cell more than 90% of the ATP is generated by aerobic processes in the mitochondria. In order to maintain the phosphorylation potential during transient hypoxia an animal could employ one or a combination of the following means. (1) Increase the glycolytic (i.e. anaerobic) ATP production to compensate for the lack of oxidative phosphorylation. Per molecule of glucose 38 molecules of ATP are generated if the glucose is fully oxidised, but only two of these are produced glycolytically. This strategy would hence require very high rates of anaerobic glycolysis and thus lead to a rapid exhaustion of carbohydrate reserves while toxic (acidic) end products would be accumulated. (2) Decrease the turnover of ATP; that is, reduce metabolic rate. (3) Employ special metabolic pathways to improve the ATP yield from substrates.

Animals adapted to transient anoxia appear to employ strategy (2), in many cases combined with strategy (3)[10,12,28]. As a consequence the tissue content of ATP and the phosphorylation potential are stabilised during hypoxia, although at somewhat lower levels than during normoxia, and basal physiological processes are maintained during the anoxic interval. The situation is different in adult insects. If insects are subjected to anoxia, energy-rich phosphates (phosphoarginine and ATP) drop to very low levels. After prolonged anoxia, ATP is hardly detectable in insect organs. This observation raises the question of which products are formed from ATP during anoxia in insects? We have followed the catabolism of ATP in organs from anoxic locusts by means of ^{31}P NMR spectroscopy and HPLC.

The content of ATP in normoxic locust flight muscle was found to be 7.63 µmol per g muscle (wet wt). Exposure of locusts to an atmosphere of pure nitrogen for 5 min did not cause a significant decrease in ATP content in muscle although, by this time, the locusts were already completely paralysed and had lost all signs of life. Hence the rapid onset of muscular paralysis cannot have been caused by shortage of ATP in muscles. Rather, anoxic failure of the nervous system appears to block muscular activity. The energy status of the CNS is much more sensitive to anoxia than that of muscle. The content of ATP in brain was reduced to 36% after 5 min of anoxia when ATP in muscle was still at its normoxic control level. After 30 min of anoxia, however, the ATP content of flight muscle was also significantly decreased, by about 20%, and it fell to less



① ATPases
② Phosphagen kinase
③ Oxidative phosphorylation Substrate-level phosphorylation
④ Adenylate kinase
⑤ 5′-Nucleotidase
⑥ Adenosine kinase
⑦ AMP deaminase
⑧ Adenylosuccinate synthase
⑨ Adenylosuccinate lyase
⑩ Adenosine deaminase
⑪ Purine nucleoside phosphorylase
⑫ Adenine phosphoribosyl transferase
⑬ Hypoxanthine phosphoribosyl transferase
⑭ Xanthine dehydrogenase
PNC Purine nucleotide cycle

Figure 2. Schematic representation of the metabolism of adenine nucleotides in animal cells. AMP is a key metabolite as it can rapidly be converted to ATP or further catabolized via IMP or adenosine. In mammals (and probably many other animals) the route for the final degradation of these two metabolites occurs via the same intermediate, the nucleoside inosine. This is converted into the base hypoxanthine by phosphorolysis. If not salvaged by reconversion to inosine, hypoxanthine will be degraded to uric acid for excretion via xanthine as intermediate. Both steps are catalyzed by the same enzyme, xanthine dehydrogenase (xanthine oxidase). The oxidase form of this enzyme is of particular interest as it can generate superoxide radicals $O_2^{-\cdot}$, which can cause tissue damage (for details see text).

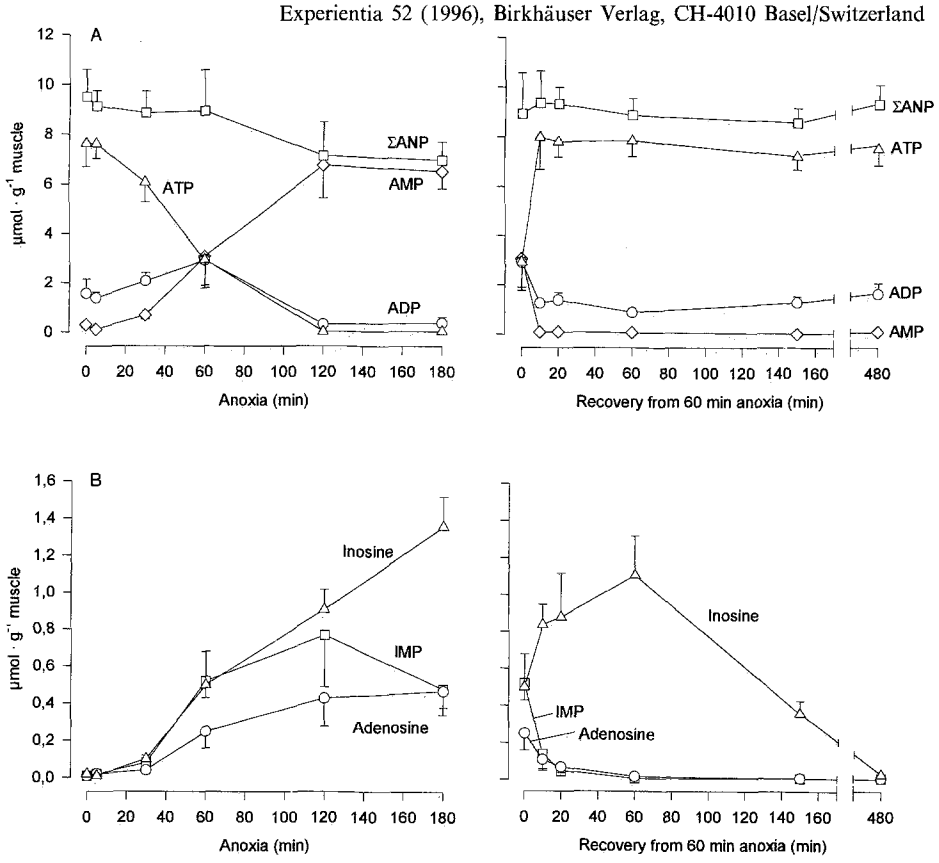


Figure 3. Metabolism of adenine nucleotides and related compounds in the flight muscle of the locust (*Locusta migratoria*) during anoxia and post-anoxic recovery. Adult male locusts, 20 to 25 days after the final molt, were subjected to an atmosphere of pure nitrogen (anoxia) at room temperature. Post-anoxic recovery was studied in locusts following 60 min of anoxia. After the experiments the locusts were submerged in liquid nitrogen to stop metabolic processes quickly[4]. Tissue samples were dissected while the insects were frozen. Tissue was extracted with perchloric acid and metabolites were measured using reversed phase HPLC.
A) ATP, ADP, AMP and total adenine nucleotides (ΣANP) during anoxia and post-anoxic recovery.
B) Products of AMP degradation (IMP, adenosine and inosine) in locust flight muscle during anoxia and post-anoxic recovery (from 60 min anoxia). For details see text.

than 1% of the normoxic value within 2 h of anoxia. ADP increased during the first hour of anoxia but was decreased to levels below the control value if anoxia was prolonged. The main product of ATP breakdown was AMP which reached levels above 6.5 µmol per g muscle after 2 h of anoxia. The total adenine nucleotide pool was little changed during 60 min of anoxia, and during 3 h of anoxia the total adenylates had decreased by 26%. Hence only a minor fraction of the AMP was degraded during 3 h of anoxia. This is in contrast to most mammalian tissues where AMP is rapidly degraded rather than accumulated. These differences between mammals and insects must be due to differences in the activity and regulation of enzymes acting in the catabolism of AMP.

In anaerobic flight muscle the catabolism of AMP gave rise to three products, adenosine, IMP and inosine (see fig. 2 and fig. 3). The major product was inosine, particularly if anoxia was maintained for more than 1 h. The time course suggests that AMP was first deaminated to IMP, then dephosphorylated to inosine (see fig. 3B). The activity of AMP deaminase in vivo (i.e. IMP formation) was low during the initial 30 min of anoxia, reached a peak between 30 and 60 min and was again reduced if anoxia was prolonged, although the content of its substrate AMP did not decrease during 3 h of anoxia (see fig. 3A, B). This indicates that the activity of AMP deaminase in anaerobic flight muscle must be controlled by factors other than the concentration of its substrate. The kinetics of AMP deaminase from locust flight muscle have not yet been studied, but regulatory properties of this enzyme from tissues other than locust flight muscle have been reported. The kinetics of AMP deaminase from mammalian muscle are very complex. The enzyme is activated by ATP and ADP and inhibited by inorganic phosphate[33] and its activity is also affected by reversible binding to cell structures such as myofibrillar proteins[21]. AMP deaminase from mammalian heart is modulated by phosphorylation[25]. AMP deaminase from crayfish tail muscle is strongly activated by ATP[24]. ATP might activate deamination of AMP also in anoxic locust flight muscle. This would be in accordance with the observation that IMP ceased to accumulate when the content of ATP had reached low levels (see fig. 3A, B).

The catabolism of adenine nucleotides in anaerobic locust flight muscle seems to end at the level of inosine. Neither hypoxanthine nor xanthine nor uric acid could

be detected in locust flight muscle during anoxia or post-anoxic recovery. There is also no indication that these compounds are formed in the anoxic brain. This is interesting because in some tissues the production of xanthine and uric acid can give rise to the formation of reactive oxygen species that have been suspected to cause tissue damage (see section: 'Anoxia and tissue damage, . . .').

Recovery from prolonged anoxia

In adult insects anoxia causes the rapid breakdown of physiological functions and an almost total loss of ATP in the tissues, resulting in a state of complete immobility similar to rigor mortis. Many insects, however, can survive this state for several hours and will recover rapidly and spontaneously if returned to air. An essential prerequisite for recovery is the resynthesis of ATP, i.e. restoration of the phosphorylation potential because this is the driving force for cellular functions.

During the recovery period, the rate of ATP synthesis must exceed that of ATP hydrolysis and this can only be achieved if oxidative phosphorylation is reactivated. Reactivating of aerobic ATP production in insects is clearly facilitated by their mode of O_2 provision. O_2 can reach the tissues via the tracheal system independently of the circulation. The increasing cellular concentration of ATP will reduce that of ADP and also of AMP via the near-equilibrium reaction catalysed by adenylate kinase (see fig. 2). The energy-rich phosphate of ATP can further be used to recover, at least partially, the products of AMP catabolism.

Adenosine is most likely rephosphorylated to AMP by adenosine kinase. The enzyme has been demonstrated to occur in locust tissue, where its activity is inhibited by AMP and ADP[2]. The rapid decrease in AMP during post-anoxic recovery could hence deinhibit the enzyme.

The concentration of IMP also decreased rapidly during post-anoxic recovery, but this was most probably due to its dephosphorylation to inosine rather than its reconversion to AMP, because the content of inosine increased while that of IMP decreased. This hypothesis is in agreement with known regulatory properties of 5′-nucleotidase, which is activated by ATP and strongly inhibited by P_i [35].

Anoxia and tissue damage, or, why are insects so tolerant of anoxia?

How the tissue damage that results from anoxia is brought about is not fully understood. The involvement of several factors and mechanisms has been proposed and there is evidence that different mechanisms contribute, depending on the tissue involved. We shall briefly discuss some factors that are related to anoxia-induced metabolism of adenine nucleotides and are likely to cause anoxic tissue damage.

1) Degradation of ATP and loss of precursors for adenine nucleotide synthesis

A breakdown of the cellular energy status is thought to have deleterious effects in mammalian organs such as brain and heart[22,23].

In the case of insects the almost total degradation of ATP in the cells is not lethal, provided it does not continue for too long. Some insects have been shown to endure extremely low ATP levels in their tissues for hours and still recover with no obvious damage. Several factors seem to contribute to this amazing capability. (A) During recovery O_2 can easily reach the tissues via tracheae without a previous reactivating of the circulation. (B) The degradation of adenine nucleotides seems not to proceed beyond the stage of inosine (see fig. 2) and relatively low amounts of adenine nucleotides are lost from insect tissue during anoxia and post-anoxic recovery.

2) Increased cytosolic Ca^{++}

Calcium is a potent physiological signal, triggering processes such as contraction, exocytosis, catabolism of carbohydrates and lipids, and degradation of proteins, phospholipids and nucleic acids. If not controlled, some of these activities could cause serious damage to the cells. Therefore, cytosolic [Ca^{++}] must be kept low, and this requires metabolic energy for either exporting Ca^{++} into the extracellular space or sequestration of Ca^{++} in cellular stores such as parts of the endoplasmatic reticulum or the mitochondrial matrix. Lack of ATP would lead to an uncontrolled increase in cytosolic [Ca^{++}] and this has long been suggested as a major cause of anoxic cell damage[11,23]. Little is known about the effects of hypoxia on Ca^{++} fluxes in insect tissues. There appear to be differences between insects and mammals with respect to mitochondrial Ca^{++} metabolism. Mitochondria from insect flight muscle have been reported to be relatively impermeable to Ca^{++} [17].

3) Anoxia-induced acidification of tissues

If ATP hydrolysis exceeds ATP synthesis, as is the case during hypoxia, potent activators of carbohydrate catabolism are generated, such as inorganic phosphate, ADP and AMP (see fig. 2). As a consequence anoxia would trigger the breakdown of glycogen reserves and a marked increase in glycolytic flux. This has been observed in mammalian organs such as brain and heart, where anoxia results in a massive accumulation of lactic acid and hence protons. Tissue acidification has long been discussed as a factor aggravating anoxic tissue damage.

Insect organs are generally dependent on aerobic ATP production and they contain relatively little lactate dehydrogenase activity. Hence, little lactic acid is pro-

duced in anoxic insect tissue. The anoxic locust brain, for instance, produces less lactic acid during 4 h of anoxia than the ischemic mouse brain does within just 2 min[27].

4) Formation of active oxygen species

Molecular oxygen can be a source of highly toxic compounds if it is involved in reactions where it is only partially reduced. These reactions seem to be reinforced in tissues when oxygen is again available after an anoxic or ischemic interval. There is good evidence that in mammals some of the tissue damage is actually not caused during anoxia but in the reperfusion period (reperfusion injury)[1,3,5,15]. It is conceivable that anoxia-induced disturbance of cellular structures leads to an increased formation of partially reduced O_2 species such as the highly reactive superoxide anion $O_2^{-\cdot}$. The superoxide anion is a free radical, i.e. it carries an unpaired valence electron (indicated by a dot) and is therefore very reactive. The superoxide radical can be eliminated by the combined action of two ubiquitous enzymes; (a) superoxide dismutase (SOD), producing hydrogen peroxide from $O_2^{-\cdot}$, and (b) catalase, removing the peroxide.

(a) $2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$

(b) $2H_2O_2 \rightarrow O_2 + 2H_2O$

However, as detoxification may not be fully effective, hydroxyl radicals $OH^{\cdot}$ can be generated (probably according to the Haber-Weiss reaction)

$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + HO^- + HO^{\cdot}$

The hydroxyl radical is even more reactive, and therefore more toxic, than $O_2^{-\cdot}$. The Haber-Weiss reaction is catalysed by iron(III) which is released from its ferritin stores[6] during ischaemic anoxia[19].

Reactive radicals are capable of attacking and splitting covalent bonds and in this process new radicals can be formed, so that chain reactions are promoted. $OH^{\cdot}$ has a strong tendency to promote chain reactions, and unsaturated lipids (as present in cell membranes), proteins and DNA are all sensitive to the attack by $OH^{\cdot}$.

Active oxygen species can be formed as a consequence of the anoxic degradation of adenine nucleotides. This has been observed in mammalian tissues where anoxia seems to induce (probably via Ca^{++}-stimulated proteolysis) the conversion of xanthine dehydrogenase (which uses NAD^+ as an electron acceptor) into xanthine oxidase[16,8]. Like xanthine dehydrogenase, xanthine oxidase oxidises both hypoxanthine and xanthine (see fig. 2) but transfers electrons (one or two) to molecular oxygen so that reactive oxygen species ($O_2^{-\cdot}$ or O_2^{2-}) will be generated. Hypoxanthine seems not to be a product of anoxic degradation of adenine nucleotides in insects, so that the anoxia-induced radical formation from this source seems unlikely. Moreover, xanthine dehydrogenase, but not xanthine oxidase, could be demonstrated in insects[9,26]. Anoxia seems unlikely to stimulate the formation of xanthine oxidase from xanthine dehydrogenase as it does in some mammalian tissues.

Conclusions and prospects

Adult insects are highly active animals, their amazing physiological capacities are based on intensive aerobic ATP production. Deprivation of oxygen causes, as it does in mammals, an almost instantaneous breakdown of physiological functions. Unlike mammals, however, insects can completely recover after many hours of anoxia. This proves that high standard metabolic rates, rapid loss of physiological functions and the breakdown of the cellular energy status do not inevitably lead to irreversible tissue damage. Adult insects are therefore interesting 'animal models' with respect to the question of which structural, physiological and metabolic properties improve the tolerance of hypoxia/anoxia. Unfortunately, our knowledge of insect anaerobiosis is still very fragmentary. This has limited our discussion to some aspects of tissue hypoxia in which insects are known to differ from mammals. More comparative studies are awaited to advance our knowledge of the biological properties and mechanisms involved in tolerance, which may eventually form the basis of methods to reduce injury from hypoxia and improve post-hypoxic recovery.

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