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Mechanisms of cell survival in hypoxia and hypothermia

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Summary

Most animals experience some degree of hypoxia and hypothermia during the course of their natural life history either as a consequence of ambient 'exposure' *per se* or through metabolic, respiratory and/or circulatory insufficiency. A prevailing experimental approach has been to probe tissues from natural models of hypoxia-tolerant and cold-tolerant vertebrates to look for common mechanisms of defence against O_2 lack and hypothermia. The ability to sustain vital cellular functions in severe cases of either condition varies widely amongst the vertebrates. Like humans, the vast majority of mammals are unable to survive prolonged periods of hypothermia or O_2 deprivation owing to irreversible membrane damage and loss of cellular ion homeostasis in vital organs such as the brain and heart. However, numerous hibernating endotherms, neonatal and diving mammals as well as many ectotherms can tolerate prolonged periods that would, in clinical terms, be called asphyxia or deep hypothermia. The key to their survival under such conditions lies in an inherent ability to downregulate their cellular metabolic rate to new hypometabolic steady states in a way that balances the ATP demand and ATP supply pathways.

Key words: hypoxia, hypothermia, metabolic depression, ion homeostasis, channel arrest, ion-motive ATPase, neurone, muscle, hepatocyte.

Introduction

Like humans, most mammals possess little naturally evolved tolerance to severe hypoxia or hypothermia, and their excitable cells and tissues are normally debilitated by any prolonged exposure to either condition. The primary causes of anoxia- or hypothermia-induced death in mammals are brain dysfunction and cardiac arrhythmias due to a loss of ionic integrity of the cell membranes. Ion leakage across cell membranes occurs as a result of both intracellular and extracellular ions drifting towards their thermodynamic equilibrium. Maintenance of a homeostatic intracellular environment therefore requires the redistribution of these ions through the use of ATP-dependent pumping systems such as the Na^+/K^+-ATPase, which can consume 20–80 % of the cell's resting metabolic rate depending on the extent of its electrical activity (e.g. muscle *versus* brain; Edwards et al., 1989; Rolfe and Brand, 1996; Priebe et al., 1996; Rolfe and Brown, 1997). Cell death occurs when ATP production fails to meet the energetic maintenance demands of ionic and osmotic equilibrium. As levels of high-energy phosphates decline, this leads to a failure of ion-motive ATPases, followed by membrane depolarization and an uncontrolled influx of Ca^{2+} through voltage-gated Ca^{2+} channels. The rise in free cytosolic intracellular Ca^{2+} concentration results in the activation of Ca^{2+}-dependent phospholipases and proteases that further hasten the rate of membrane depolarisation, leading to uncontrolled cellular swelling and, ultimately, to cell necrosis (Hochachka, 1986; Fig. 1). The single most protective and unifying feature of cold-tolerance in hibernating and neonatal mammals is the same one that ensures their enhanced survival in O_2-limiting conditions: a regulated metabolic depression. In contrast, the forced suppression of metabolism in largely aerobic, cold-sensitive animals is, in effect, metabolic failure. Whereas the time course of the latter is metered in minutes to hours, the ability to depress ATP turnover in a regulated fashion effectively delays the onset of cell death by stabilising concentrations of adenylates at new hypometabolic steady states for periods of hours or days (Fig. 1).

In cases of severe O_2 limitation, most excitable cells of mammals cannot continue to meet the energy demands of active ion-transporting systems, leading to rapid exhaustion of fermentable substrate, catastrophic membrane failure and cell death. However, in certain lower vertebrates, neonates and diving mammals, hypoxia-induced membrane destabilisation of the kind seen in adult mammals is either slow to develop or does not occur at all as a result of adaptive decreases in membrane permeability (i.e. ion 'channel arrest') that dramatically reduce the energetic costs of ion-balancing ATPases (Hochachka, 1986). However, the hypothermia-induced mismatch between ATP supply and demand immediately preceding the forced hypometabolism of cold-sensitive animals (Fig. 1) is thought to occur as a result of metabolic imbalances caused by the differential

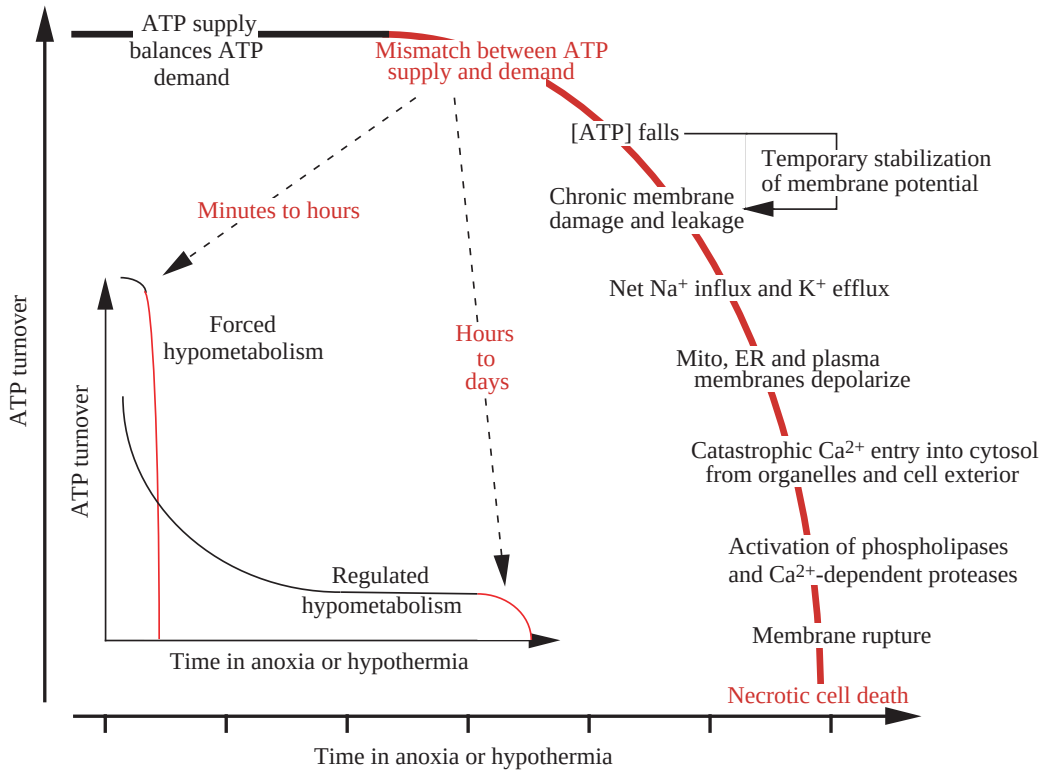

Fig. 1. ATP turnover of cells as a function of time exposed to anoxia or hypothermia. The main figure shows the debilitating cascade of events leading to necrotic cell death. When cellular ATP demand exceeds ATP supply, this cascade is the same (in general if not in specific detail) whether the cells are 'anoxia- or cold-sensitive' or 'anoxia- or cold-tolerant'. The inset shows that a regulated suppression of ATP turnover (i.e. a regulated hypometabolism in which ATP demand balances ATP supply) extends the time to the onset of the debilitating cascade in 'anoxia- and cold-tolerant cells'. In contrast, an early mismatch between ATP supply and demand in 'anoxia- and cold-sensitive cells' leads to a forced hypometabolism which is, in effect, early metabolic failure. Mito, mitochondria; ER, endoplasmic reticulum.

effects of temperature on the rates of independent ATP supply and ATP demand pathways (Hochachka, 1986). There is no reason to suspect, for example, that the metabolic rates of all organ systems should exhibit a uniform thermal dependency, since metabolic pathways are often not only specific to particular tissues but may express unique Q_{10} responses to forced hypothermia (Hochachka and Somero, 1984). This could explain why hypothermia-induced failure of the respiratory system precedes that of cardiovascular collapse in some cold-sensitive endotherms (Willis, 1979; Musacchia, 1984). In contrast, tissue-specific metabolic pathways in cold-tolerant animals are expected to display more uniform thermal dependencies that facilitate the balanced suppression of the ATP supply and ATP demand pathways (Fig. 1).

Cellular responses to O_2 lack

Animals are broadly classified according to their metabolic responses to hypoxia either as (i) oxyregulators, whose metabolism exhibits a high obligatory rate of energy consumption, or (ii) oxyconformers, whose energy demands decrease with decreasing availability of O_2. These broad classifications also apply to individual tissues and organs, with oxyregulating brain being generally regarded as the most hypoxia-sensitive organ and skeletal muscle amongst the most hypoxia-tolerant. As O_2 flow to tissues decreases, the cellular ATP demands of most mammalian cells and tissues tend to remain constant, leading to an energetic deficit that can be made up for only by activation of anaerobic ATP supply pathways (the so-called 'Pasteur effect'). However, anaerobic ATP production cannot sustain the pre-existing energy demands of mammalian cells and tissues for more than a few minutes (in the case of the brain) or hours (in the case of muscle) because of the rapid depletion of fermentable substrate together with the accumulation of deleterious end-products (e.g. H^+). At the other end of the anoxia-tolerance spectrum are animals such as the crucian carp (*Cyprinus carpio*), the common frog (*Rana temporaria*) and the freshwater turtle (*Chrysemys picta bellii*), all of which routinely survive periods of O_2 deprivation that would spell certain death to most mammals (Wegener and Krause, 1993; Lutz and Nilsson, 1997; Boutilier et al., 1997). Their naturally evolved tolerance to anoxia can be attributed to an unusually well-developed capacity for rapid entry into, and return from, metabolically depressed steady states. Indeed, these so-called 'facultative vertebrate anaerobes' use anaerobic metabolism not to maintain pre-existing rates of

ATP production but to sustain reduced rates of energy turnover during hypoxia (Fig. 1, inset). The net effect of this regulated metabolic depression is that it conserves fermentable fuel, reduces deleterious end-product formation and extends survival time. By comparing anoxia-tolerance across phylogenetic lines, the hope is to identify mechanisms of protection against anoxia/ischaemia that animals have in common.

ATP-consuming processes in cellular respiration

At the cellular level, metabolic depression may be brought about (i) by decreasing energy-consuming processes and/or (ii) by increasing the efficiency of energy-producing pathways (for reviews, see Hochachka and Guppy, 1987; Storey and Storey, 1990; Hochachka et al., 1996; Brand et al., 2000; Boutilier and St-Pierre, 2000). Protein synthesis and ion-motive ATPases are the dominant energy-consuming processes of cells at standard metabolic rate (SMR; Rolfe and Brown, 1997), so decreases in the ATP demand of these processes at minimal metabolic rate (MMR) are thought to be largely responsible for enabling the downregulation of energy turnover in metabolically depressed states (Guppy et al., 1994; Hand and Hardewig, 1996; Hochachka et al., 1996; Donohoe et al., 2000). Less is known about the metabolic efficiency of the processes that make up SMR in vertebrates. These involve stoichiometric efficiencies that maximize the molar ratios of ATP to O_2 during hypoxia or of ATP to H^+ during fermentation (Hochachka and Somero, 1984; Brand et al., 2000). Given that SMR represents the starting point against which metabolic reductions are compared, it is imperative that the reactions that constitute SMR are well defined. Approximately 20 % of mammalian whole-animal O_2 consumption can be attributed to mitochondrial proton leak and non-mitochondrial respiration (Brand et al., 2000). Of the 80 % of SMR that is coupled to ATP synthesis, 25–30 % is used by protein synthesis, 19–28 % by the Na^+/K^+-ATPase, 4–8 % by the Ca^{2+}-ATPase, 2–8 % by the actinomyosin-ATPase, 7–10 % by gluconeogenesis, 3 % by ureagenesis, with substrate cycling and mRNA synthesis making significant contributions (for a review, see Rolfe and Brown, 1997). Thus, protein synthesis and ion-motive ATPases are the dominant ATP-consuming processes in cells, making up more than 90 % of the oxidatively coupled ATP consumption of rat skeletal muscle (Rolfe and Brand, 1996) and as much as 66 % of the ATP turnover in rat thymocytes (Buttgereit and Brand, 1995).

A number of cells and tissues of facultative anaerobes have become particularly useful models for the study of cellular energetics since they possess an intrinsic capacity for anoxia-induced hypometabolism. For example, the metabolic rates (measured as heat flow using micro-calorimetry) of turtle hepatocytes (Buck et al., 1993a), turtle brain cortical slices (Doll et al., 1994), frog skeletal muscle (West and Boutilier, 1998) and frog heart fall to approximately 25 % of the normoxic rates after only 30 min of exposure to anoxia (Fig. 2). Reoxygenation reverses these effects rapidly and completely. Moreover, intracellular ATP concentrations remain stable throughout the cycles of anoxia and reoxygenation, consistent with the maintained energy balance observed in the tissues of animals exposed to O_2 lack *in vivo* (Wegener and Krause, 1993; Donohoe and Boutilier, 1998; Donohoe at al., 1998). Since protein turnover and ion-motive ATPases are major ATP sinks in many cells and tissues at SMR (Rolfe and Brown, 1997), these energy-consuming processes represent primary cellular targets for downregulating ATP demands in response to O_2 lack.

One anoxic-defence mechanism shared across phylogenetic lines is the ability to reallocate cellular energy between essential and non-essential ATP demand processes as energy supplies become limiting. Studies on both rat and turtle hepatocytes show that protein synthesis is largely inhibited in response to anoxia (Buc-Calderon et al., 1993; Land and Hochachka, 1994; Land and Hochachka, 1995). A prevailing theory is that the energy spared by the reduction in protein synthesis is reallocated to more critical cell functions involved in osmotic and ionic homeostasis. Indeed, when cellular energy supplies are progressively inhibited in rat thymocytes, ATP-consuming processes less essential for the immediate survival needs of the cell are given up before those more critical for maintaining ionic integrity (Buttgereit and Brand, 1995). In effect, the ATP-consuming processes are arranged in a hierarchy, with protein synthesis and RNA/DNA synthesis falling off rather sharply as energy becomes limiting, and with Na^+/K^+ pumping and Ca^{2+} cycling taking the greatest priority. This implies that ion-motive ATPases are likely to become the dominant energy sinks in anoxic mammalian cells, just as the Na^+/K^+ pump does in the anoxic turtle hepatocyte (Hochachka et al., 1996).

Channel arrest versus *channel leak*

The critical difference between anoxia-tolerant and anoxia-sensitive animals is that the latter show no reduction in the absolute ATP demand of the ion-motive ATPases in response to O_2 lack. In contrast, cells of anoxia-tolerant animals exhibit large-scale reductions in absolute Na^+/K^+ activity during anoxia. Although this might normally be interpreted as pump failure in a mammalian cell, such decreases in Na^+/K^+-ATPase activity in the brain, liver and muscle cells of facultative anaerobes are brought about without any disruptions in electrochemical potentials, cellular ion levels or ATP concentrations (Buck et al., 1993b; Buck and Hochachka, 1993; Lutz and Nilsson, 1997; Bickler and Buck, 1998; West and Boutilier, 1998; Donohoe et al., 2000). Thus, quite apart from there being any failure of ionic homeostasis, the reduction in Na^+ pump activity in anoxia-tolerant cells is part of a coordinated process of energy conservation wherein O_2 lack initiates a generalised suppression of ion-channel densities and/or channel leak activities. The net result is that cell membrane permeabilities are reduced, thereby lowering the energetic costs of maintaining transmembrane ion gradients. This phenomenon, so-called ‘channel arrest’ (Hochachka, 1986), serves as a potent mechanism for actively downregulating the ATP demands of cells in potentially

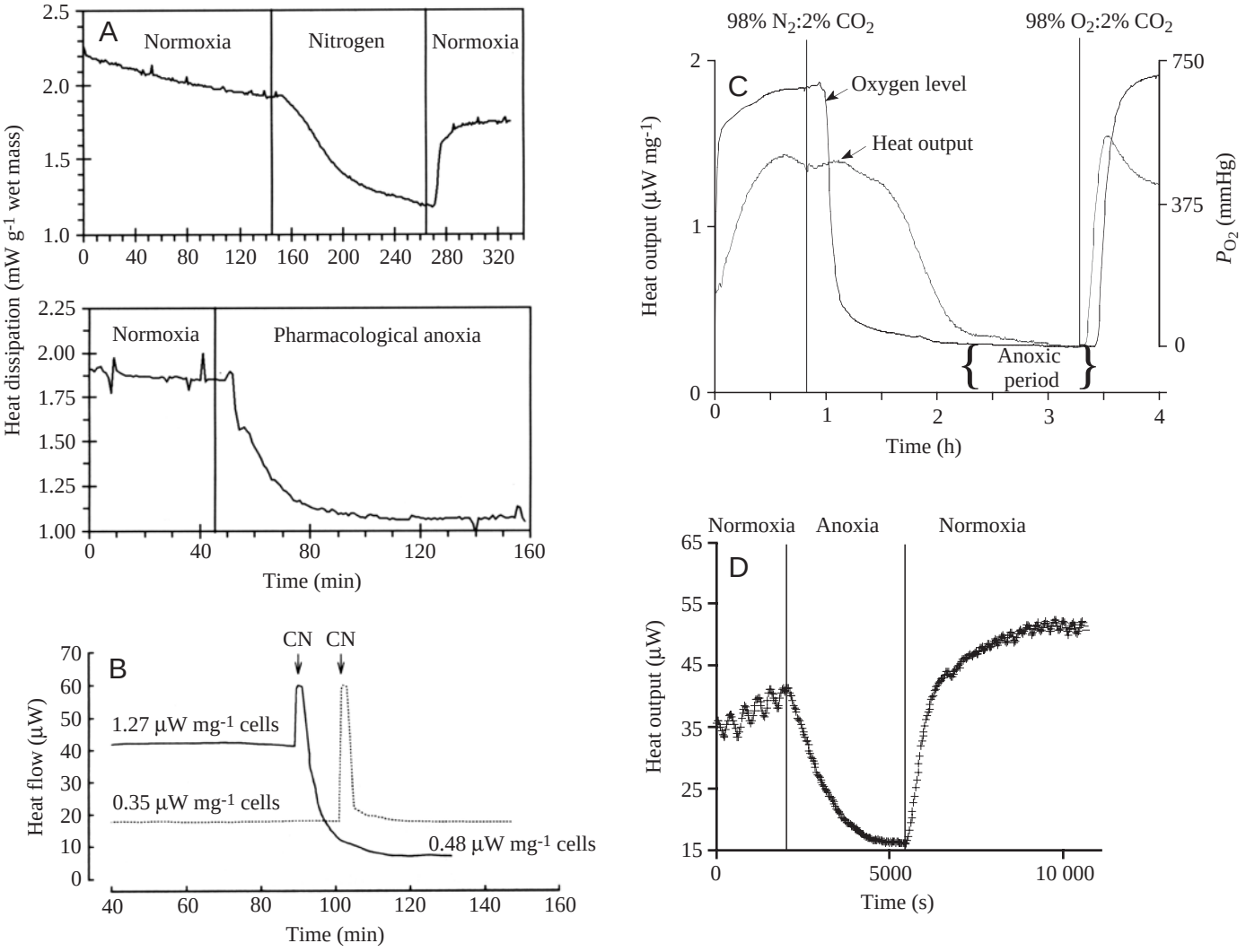

Fig. 2. Recordings of heat dissipation showing the metabolic depression response to anoxia in (A) turtle brain cortical slices incubated in oxygen-free medium (nitrogen) or in the presence of sodium cyanide (pharmacological anoxia) at 25 °C (Doll et al., 1994), (B) turtle hepatocytes, where CN denotes addition of sodium cyanide to cell preparations incubated in oxygen-free medium (dotted line) or normoxic medium (solid line) at 25 °C (from Buck et al., 1993a, with permission from the American Physiological Society), (C) frog sartorius muscle exposed to a progressively developing oxygen-free superfusate at 20 °C (from West and Boutilier, 1998, with permission from Springer) and (D) frog heart ventricular ring (20 mg) exposed to a progressively developing oxygen-free superfusate at 20 °C (T. G. West and R. G. Boutilier, unpublished data). Turtle, *Chrysemys picta belli*; frog, *Rana temporaria*. 1 mmHg=0.133 kPa.

energy-limited states. In contrast, anoxic mammalian cells, particularly in brain and heart, show all the hallmarks of a non-adaptive 'channel leak' response. For example, hypoxia-induced increases in the persistent Na^+ current in intact cardiac (Ju et al., 1996) and hippocampal (Hammarström and Gage, 1998) cells as well as corresponding increases in amiloride-sensitive Na^+/H^+ and Na^+/Ca^{2+} exchange activity in nerve cells (Chidekel et al., 1997; Haddad and Jiang, 1997) lead to increases in transmembrane Na^+ cycling. This evidently raises the ATP demand of ion-balancing ATPases to breaking point, which triggers abnormal accumulation of Na^+, leading to deleterious Ca^{2+} overload and cell damage and/or death (Hammarström and Gage, 1998; Fig. 1).

Ion channel arrest has been studied most extensively in models of anoxia-tolerant brain, where it is considered to be one of the most important neuroprotective mechanisms against O_2 lack (Lutz and Nilsson, 1997; Bickler and Buck, 1998). One of the tenets of the 'channel arrest theory' is that ion-channel densities should be inherently lower in hypoxia-tolerant animals than in their hypoxia-sensitive counterparts (Hochachka, 1986). Although this appears to be the case for Na^+ channel density both for turtle cerebellum (Xia and Haddad, 1991) and in isolated nerve endings of turtle brain (i.e. synaptosomes; Edwards et al., 1989), it cannot in itself explain the 100-fold difference in anoxia-tolerance between turtle and rat brain. Unlike their mammalian counterparts, ATP levels and ionic gradients in turtle neurons are maintained perfectly homeostatic during anoxia, thereby avoiding the fatal

consequences of energy failure (Fig. 1). The remarkable anoxia-tolerance of the brain cells of these facultative anaerobes appears to be effected through decreased electrical activity (Sick et al., 1982; Feng et al., 1988) and reduced rates of transmembrane ion leakage (channel arrest) (Lutz et al., 1985; Hand and Hardewig, 1996). This downregulation of firing rates and synaptic transmission suppresses cellular metabolism by 50–80 % (Doll et al., 1994; Lutz and Nilsson, 1997). In turtle brain, anoxia-induced suppression of current flow through Na^+ channels also leads to the elimination of action potentials ('spike arrest'), thereby reducing the energetic costs of neurotransmission and decreasing the ATP demands of the ion pumps involved in the maintenance of electrochemical gradients (Sick et al., 1993). Although decreases in neuronal Na^+ channel density of turtle brain have been observed in response to O_2 lack (Peréz-Pinzón et al., 1992a; Peréz-Pinzón et al., 1992b), the mechanisms of Na^+ channel recycling from membrane to cytosol and of probable changes in leak activity at the level of the channel itself remain elusive (Bickler and Buck, 1998).

Putative modulators of anoxia-induced channel arrest

Little is known about the signalling pathways involved either in channel arrest or in the transition to hypometabolic states during anoxia. Although there has been some speculation that decreased intracellular pH and/or increased CO_2 partial pressures may trigger metabolic rate depression (Lutz, 1989; Wasser et al., 1990), the mechanisms remain elusive. While there are many other possible systemic co-mediators of metabolic rate (e.g. cortisosteroids, insulin, glucagon, thyroid hormones), there has been intense interest of late in more local chemical triggers. As the initial end-product of ATP degradation, adenosine could offer a direct measure of cell metabolic status through its receptor systems (Belardinelli and Shryock, 1992). For example, concentrations of adenosine are inversely proportional to energy charge in the isolated heart of the frog *Rana ridibunda* (Lazou and Beis, 1986). In such a case, adenosine would be an obvious candidate for signalling when rates of ATP synthesis fall below rates of ATP usage. Indeed, marked increases in extracellular adenosine concentration are thought to play an important neuroprotective role during the initial stages of energy failure in anoxic and ischaemic mammalian brain (Hagberg et al., 1987; Newby et al., 1990). Adenosine is also released into the extracellular space of turtle brain shortly after the onset of anoxia (Nilsson and Lutz, 1992; Lutz and Nilsson, 1993; Pék and Lutz, 1997). Moreover, when anoxic isolated turtle cerebellum is superfused with adenosine receptor blockers, rates of K^+ efflux increase and thereby hasten the onset of membrane depolarisation (Pék and Lutz, 1997). That adenosine itself may be involved in coordinating metabolic and ion-channel arrest seems well worth pursuing since, at least in brain, it is known to reduce cell excitability, to stimulate glycogenolysis and to increase cerebral blood flow (Magistretti et al., 1986; Morii et al., 1987; Lutz and Nilsson, 1997).

Because the Na^+/K^+-ATPase ion pump is the primary ATP-consuming process in neurons, its failure as a result of anoxia-induced energy shortages in largely aerobic animals leads to a rapid dissipation of the pre-existing Na^+ gradient. This causes the Na^+/glutamate cotransporter to reverse direction, so that instead of the cell taking up glutamate following its synaptic release, glutamate accumulates in the extracellular space. Because glutamate is a major excitatory neurotransmitter, its extracellular accumulation in anoxia-sensitive brain hyperactivates glutamate receptors and leads to excitotoxic injury. In contrast, glutamate is not released by the turtle brain during anoxia (Nilsson and Lutz, 1991). Moreover, glutamate receptor activity decreases when turtle brain is exposed to hypoxia, stabilising intracellular [Ca^{2+}] and thereby promoting cell survival (Bickler and Buck, 1998). However, increases in levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and in GABA receptor numbers in anoxic turtle brain (Nilsson and Lutz, 1991; Nilsson and Lutz, 1993) may facilitate the suppression of neurotransmission and the overall depression of brain electrical activity that accompanies metabolic depression.

Mitochondrial responses to O_2 lack: cellular treason

When sufficient oxygen is available for oxidative phosphorylation, the mitochondrial F_1F_o-ATPase acts as the site for ATP production in animal cells. During anoxia, however, this reversible enzyme, the ATP synthase, begins to run backwards as it actively pumps protons from the matrix in an attempt to maintain the mitochondrial membrane potential (Fig. 3). Thus, during anoxia and ischaemia, mitochondria change from being ATP producers to potentially powerful ATP consumers (Lisa et al., 1998). This occurs at the very time the anoxic cell needs to limit ATP use, not enhance it. As discussed above, numerous mechanisms of energy conservation are marshalled into action when cells are exposed to O_2 lack. Species that can survive prolonged periods of O_2 lack must therefore limit ATP use by the F_1F_o-ATPase during anoxia; otherwise, this process would dominate glycolytic metabolism and threaten ATP delivery to the essential ATP-consuming processes of the cell (e.g. ion-motive ATPases). Two ways in which ATP hydrolysis by the F_1F_o-ATPase might be limited during anoxia are (i) by a reduction of the proton conductance of the mitochondrial inner membrane and/or (ii) by inhibition of the enzyme. St-Pierre et al. (St-Pierre et al., 2000) assessed these two possibilities using intact mitochondria isolated from the skeletal muscle of anoxia-tolerant frogs. Whereas proton conductance appeared to be unaltered upon exposure to anoxia, ATP use by the F_1F_o-ATPase is limited during anoxia by a profound inhibition of the enzyme.

An F_1-ATPase inhibitory subunit (IF_1), first discovered by Pullman and Monroy (Pullman and Monroy, 1963), is known to inhibit the hydrolysis of ATP by mitochondria in a pH-dependent manner during anoxia. IF_1 binds to the mitochondrial ATPase at low pH under non-energising conditions in a way that reduces its rate of ATP utilisation (for a review, see Rouslin, 1991). 'Class a' species, which include

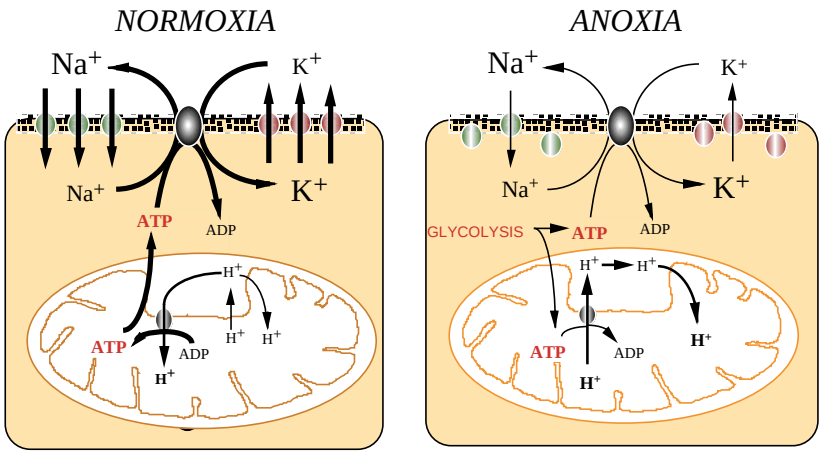

Fig. 3. A generalised model of cell membrane 'channel arrest' and mitochondrial membrane 'H^+-ATPase activation' in response to anoxia. In this model, anoxia-induced decreases in Na^+ and K^+ channel densities (and associated ion-channel activities) lead to a net reduction in Na^+/K^+-ATPase activity, thereby lowering the ATP demand for maintaining transmembrane ion concentration gradients. At the level of the mitochondria, oxidative phosphorylation during normoxia occurs when protons are transferred across the inner mitochondrial membrane (at complexes I, III and IV), thereby generating a proton-motive force that provides the driving force for proton influx through the F_1F_o-ATPase (also known as ATP synthase). Proton influx apparently drives the ATP synthase to phosphorylate ADP to ATP. At standard metabolic rate (SMR) during normoxia, a significant fraction of the protons pumped out of the respiratory chain leak back into the mitochondrial matrix without synthesizing ATP (i.e. effectively uncoupling mitochondrial oxygen consumption from ATP synthesis). This futile cycle of mitochondrial proton pumping and proton leak across the inner mitochondrial membrane is estimated to make up approximately 20 % of the SMR of mammals (Rolfe and Brown, 1997; Brand et al., 2000). In the absence of oxygen, proton transfer no longer occurs at complexes I, III and IV, but the inverse operation of the F_1F_o-ATPase attempts to maintain the mitochondrial membrane potential by using ATP to translocate protons into the intermembrane space.

rabbit and pig, contain a full complement of higher-affinity IF_1 and display considerable inhibition of their mitochondrial ATPase during anoxia and/or ischaemia. 'Class b' species, which include rat and mouse, contain small amounts of higher-affinity IF_1 and show very little ATPase inhibition during ischaemia. Finally, 'class c' species, which include guinea pig, pigeon, turtle and frog, contain a full complement of a lower-affinity form of IF_1 and manifest a low-to-moderate inhibition of the ATPase during ischaemia (Rouslin and Broge, 1990; Rouslin et al., 1995). However, a more recent study by Rouslin and Broge (Rouslin and Broge, 1996) has shown that 'class b' and 'class c' species seem to resist better the ATP depletion during ischaemia in the absence of uncoupler than do 'class a' species. Therefore, it seems that species that lack functional IF_1 must rely on different mechanisms, yet unknown, to reduce ATP use by their mitochondrial ATPase during ischaemia. One major step forward will be to investigate whether anoxia-tolerant species reduce their rate of ATP use by the F_1F_o-ATPase during O_2 lack as part of a concerted effort to reduce energy expenditure and, if so, to elucidate the mechanism behind the inhibition. Moreover, additional studies need to be performed on cell energy budgets during anoxia, as well as on anoxic tissues with different mitochondrial densities, to determine the impact that mitochondrial ATP consumption has on the ability to preserve cellular ionic and osmotic integrity.

Because the rate of ATP use by the F_1F_o-ATPase decreases to a new steady state when frog skeletal muscle mitochondria are exposed to anoxia, this leads to the maintenance of a lower mitochondrial membrane potential (St-Pierre et al., 2000). Although this probably means that anoxic mitochondria have a reduced capacity to transport substrates compared with their normoxic counterparts, the energy saved by reducing the membrane potential (to lower but biologically viable values, rather than maintaining it at pre-existing levels) may be critical to the preservation of cell membrane potential (Fig. 3). Mitochondrial membrane potential cannot be abolished completely during anoxia since it is crucially needed to import proteins that ensure mitochondrial maintenance. Moreover, the disruption of mitochondrial membrane potential during anoxia would lead to a series of events culminating eventually in cell death by apoptosis (Kroemer et al., 1998).

It is important to note that backwards operation of the ATP synthase occurs only during anoxia and/or ischaemia since even trace amounts of oxygen can make a vital energetic contribution to aerobic ATP production. Indeed, recent studies show that mitochondrial oxidative phosphorylation is more efficient at low O_2 tensions (0–1.1 kPa) than at air saturation as a result of depressed proton leak and uncoupled respiration (Gnaiger et al., 2000).

Oxygen sensing and molecular adaptation to hypoxia

The main emphasis of this review has been on metabolic- and membrane-coupled responses to anoxia and/or ischaemia. However, many cells and tissues exhibit a whole host of adaptive molecular-level responses to sustainable levels of hypoxia, as might be typified by ascent to altitude or by chronic hypoxia due to ventilatory or circulatory insufficiency. Although the anoxia-induced 'metabolic arrest' strategy of facultative anaerobes is clearly not an option taken up by the cells and tissues of mammals at times of severe O_2 shortage, mammalian liver and heart cells can suppress their metabolic rate by up to 50 % during sustainable episodes of hypoxia (Schumacker et al., 1993; Budinger et al., 1996). In these cases, the metabolic suppression develops over 2–3 h and occurs at oxygen partial pressures (P_{O_2}) well above the critical P_{O_2} (P_{crit}) at which diffusion of oxygen to the mitochondria should begin to limit oxidative phosphorylation. This hypometabolic

response to chronic hypoxia is particularly well characterised in mammalian heart, in which decreases in myocardial oxygen delivery result in decreased contractile activity and O_2 consumption in a phenomenon termed 'hibernating myocardium' (Heuch, 1998). Other adaptive responses to hypoxia include alterations in neurotransmitter release, ion-channel activation and gene expression (Bunn and Poyton, 1996; Haddad and Jiang, 1997; Budinger et al., 1998). The bulk of evidence to date suggests that the mechanisms underlying such protection are marshalled into action by a universal but poorly understood cellular O_2-sensing pathway whose putative members include cytochromes, mitochondria and reactive oxygen species (Bunn and Poyton, 1996; Hochachka et al., 1996; Ehleben et al., 1998; Semenza, 1999).

There are now many examples of hypoxia-induced induction of genes, including those for (i) erythropoietin (EPO) and vascular endothelial growth factor (VEGF), both of which are involved in enhancing O_2 delivery to tissues, (ii) glycolytic enzymes involved in anaerobic ATP supply pathways, and (iii) heat-stress proteins (HSPs), whose expression constitutes a ubiquitous intracellular protective response to stress. There is circumstantial evidence that the universal sensor is a haem protein embodied within a multi-subunit assembly that contains a NAD(P)H oxidase capable of producing reactive oxygen species (ROS). The ROS act as signalling molecules in a cascade that normally inhibits the activation of hypoxia-inducible factor 1 (HIF-1) by mediating the degradation of HIF-1a subunits in the proteosome. The model predicts that a decrease in ROS production occurs during hypoxia, or when cobalt–haem binding mimics hypoxia, and that this decrease in levels of ROS leads to the activation of an HIF-1 heterodimer that is required for the induction of hypoxia-responsive gene expression (Bunn et al., 1998). Although there have been intense efforts to identify the various response elements of this putative O_2-sensing pathway, the underlying mechanisms of cellular O_2 sensing and regulation of the activity of HIF remain elusive (Zhu and Bunn, 2001). Others suggest that mitochondria themselves can act as O_2 sensors by increasing ROS production during hypoxia since cells depleted of their mitochondrial DNA not only fail to produce ROS in response to hypoxia but lose the ability of their wild-type counterparts for hypoxia-induced gene expression (Chandel et al., 1998). Clearly, full elucidation of the cellular O_2-sensing and signal-transduction pathways represents a major challenge for the future.

Cellular responses to hypothermia

In mammals, the term hypothermia is normally used to define either (i) a voluntary decline in core body temperature (T_b) caused by a regulated decrease in the central nervous system thermoregulatory set-point temperature, or (ii) an involuntary decline in body temperature when internal or external conditions 'force' the central set point to deviate from normal (Clark and Fewell, 1996). 'Voluntary hypothermia' therefore represents regulation around a shifted set point, rather than a failure of homeostasis, and is the characteristic response observed in mammalian and avian torpor and mammalian hibernation and during acute hypoxaemia in newborns and adults of various endothermic species (Singer and Bretschneider, 1990; Singer, 1999). Unlike endotherms, core body temperatures in ectotherms are governed principally by external rather than internal sources of heat. Thus, rather than having a fixed 'set-point' temperature, these animals exhibit 'preferred' body temperatures that can be controlled to some extent by behavioural and physiological adjustments. The preferred body temperatures of most ectotherms are generally found to be lower in winter than in summer as a result of seasonally induced acclimatory responses that effectively reset thermal tolerance ranges and lower lethal temperatures (Hochachka and Somero, 1984). So, whereas a summer-acclimatized animal with a preferred temperature of 20 °C might be considered hypothermic if acclimated and/or forced to winter temperatures of 4 °C, a winter-acclimatized animal with a preferred temperature of 4 °C would not. In contrast, when exposed to stressors such as hypoxia or hypercapnia, many ectotherms exhibit a behavioural hypothermia response, i.e. a voluntary decrease in the pre-existing preferred body

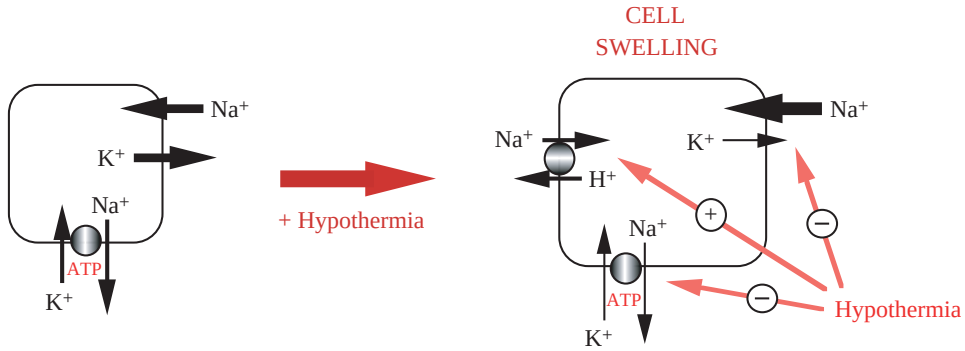

Fig. 4. Model of the hypothermia response seen in rat brain glial cells. Hypothermia inhibits the Na^+/K^+-ATPase and also upsets the normal balance between Na^+ influx and K^+ efflux in favour of Na^+ influx. This leads to a net accumulation of Na^+ that is exacerbated by hypothermia-induced activation of the Na^+/H^+ exchanger, leading to cell swelling (redrawn in part from Plesnila et al., 2000, with permission of Cambridge University Press). Further details are given in the text.

temperature (Wood, 1995) analogous to the voluntary hypothermia responses seen in many endotherms. Indeed, many animals across broad phylogenetic lines exploit hypothermia as a form of 'natural therapy' against O_2 lack. In these cases, reduction in body temperature is a regulated process that ensures a balance between ATP supply and demand.

Ionic integrity of cells during hypothermia

To understand why many mammalian tissues and cells are so cold-sensitive, we must again turn to the ion-regulatory mechanisms at the cell membrane. As with hypoxia-induced membrane destabilisation (Fig. 1), deep hypothermia in non-hibernating mammals leads to marked disturbances in cellular ion homeostasis that may be further influenced in the cold by alterations in membrane fluidity (Kruuv et al., 1983; Zachariassen, 1991; Stefanovich et al., 1995). For example, rat ventricular cells at 10 °C show a greater than 50 % increase in $[Ca^{2+}]_i$ compared with those at 30 °C, supporting the idea that intracellular Ca^{2+} overload takes place in cardiac myocytes of non-hibernating mammals during deep hypothermia (Wang and Zhou, 1999). Wang and co-workers (Wang et al., 1997) also determined the relationship between cardiac hypothermia-tolerance and the sources of activator Ca^{2+} by modifying sarcoplasmic reticulum function in papillary muscles from the cold-sensitive rat and the cold-tolerant hibernating ground squirrel. Rat muscle preparations showed a marked increase in resting tension and aftercontraction and became inexcitable as temperature was lowered from 25 to 7–12 °C. In contrast, muscle preparations from the ground squirrel maintained contractility down to 3–5 °C without aftercontraction or increased resting tension. Depressed Ca^{2+} influx evidently helps to prevent hypothermic Ca^{2+} overload of the cardiac cells of the hibernating mammal (Wang et al., 1997).

Early recognition of the deleterious effects of cold on the ionic integrity of cells from non-hibernating mammals came from studies on dogs (Thauer and Brendel, 1962) showing that deep hypothermia (T_b<15 °C) causes massive brain swelling, with accumulation of Na^+ in brain parenchyma and cellular depletion of K^+. This so-called 'cold swelling' of brain may result from a mismatch between active and passive membrane transport processes, leading to membrane depolarization and irreversible injury to cells (Hochachka, 1986; Singer and Bretschneider, 1990). If, for example, the temperature coefficient for K^+ channel efflux were significantly lower than that for ATP-dependent active accumulation by the Na^+/K^+ pump, passive diffusion processes would dominate at low temperatures. Alternatively, cellular swelling and membrane destabilisation during deep hypothermia could be caused by differential permeabilities of the major ions themselves, with little or no direct involvement of the Na^+/K^+-ATPase (see below; Plesnila et al., 2000). Ionic disruptions of the kind seen in brain can lead to dissipation of the membrane potential in other cell types of non-hibernating mammals (Wang and Wojowyk, 1988). For example, when vascular smooth muscle cells from the rat were incubated for 48 h at 7 °C, intracellular $[K^+]$ decreased by 138 mmol l^{-1} and cytosolic $[Na^+]$ increased by 148 mmol l^{-1}, with half-times of 14 and 11 h respectively. In contrast, smooth muscle cells from hibernating ground squirrels were able to maintain their ionic integrity when incubated under identical conditions (Kamm et al., 1979). Even so, the depth of hypothermia exposure is critical, since the vascular smooth muscle of both the rat and ground squirrel was able to maintain K^+ content at 17 °C (Kamm et al., 1979).

Recent studies on brain glial cells from 1- to 3-day-old rat pups show that the primary cause of cellular swelling during both mild (32 °C) and moderate (27 °C) hypothermia is intracellular accumulation of Na^+ (Plesnila et al., 2000; Fig. 4), the effects of which can be reversed completely by incubation of hypothermic cells in a Na^+-free medium (i.e. choline chloride substituted for NaCl). The Na^+/K^+ pump is thought to be largely inhibited by the cold either as a result of the direct thermodynamic effects of decreased ATP production (Singer and Bretschneider, 1990) or of the progressive development of a hypothermia-induced mismatch between ATP supply and ATP demand pathways (Hochachka, 1986). In glial cells from newborn rat pups, the Na^+/K^+ pump is thought to have no direct involvement in the cold-induced rise in intracellular $[Na^+]$. For example, blockade of the Na^+/K^+-ATPase with the drug strophanthidin, which mimics its hypothermia-induced inhibition, has no effect on the membrane potential of glial cells or astrocytes at 37 °C or during hypothermia (Plesnila et al., 2000). Instead, the cold-induced accumulation of Na^+ is evidently caused by a differential effect of cooling on the passive movements of Na^+ and K^+ across the cell membrane (Fig. 4). Cell volume and membrane potential are unaffected during inhibition of the Na^+/K^+-ATPase with ouabain since the compensatory changes in Na^+ influx and K^+ efflux occur in a 1:1 ratio. During strophanthidin- or hypothermia-induced inhibition of the Na^+ pump, however, the differential effects on Na^+ and K^+ permeability favour Na^+ influx (Dipolo and Latorre, 1972; Plesnila et al., 2000). Cold-induced acceleration of Na^+/H^+ exchange is an additional route for Na^+ influx (Fig. 4) since inhibition of the antiporter with amiloride effectively attenuates the hypothermia-induced swelling of glial cells (Plesnila et al., 2000). However, whereas this antiporter is normally activated by intracellular acidification, decreases in temperature are well known to raise rather than lower intracellular pH in most animal cells (Aickin and Thomas, 1977; Boutilier et al., 1987). A possible explanation of this apparent paradox comes from studies by Marjanovic et al. (Marjanovic et al., 1998) showing that the activation curve of the Na^+/H^+ antiporter in frog skeletal muscle is shifted to more alkaline values in the cold.

Although Na^+ accumulation appears to be the primary driving force for hypothermia-induced cell swelling in a variety of cells and tissues, the direct effects of cold on the other ion exchangers and cotransporters involved in cell volume regulation are poorly understood. Cold-induced Cl^- influx and K^+ efflux (Willis, 1979; Hochachka, 1986) imply that other ion-translocating mechanisms, such as K^+/Cl^- cotransport, $Na^+/K^+/2Cl^-$ cotransport and Cl^-/HCO_3^-

exchange, might also be involved in the hypothermia-induced swelling response. In any case, if cold-induced accumulation of Na^+ continues unabated, the rise in cytosolic [Na^+] will ultimately lead to membrane depolarisation, the opening of voltage-dependent Ca^{2+} channels, rapid influx of Ca^{2+} and initiation of membrane phospholipid hydrolysis (Hansen, 1985; Hochachka, 1986). Deleterious increases in cytosolic [Ca^{2+}] may arise through a cold-induced breakdown of plasma membrane Na^+/Ca^+ exchange, by an imbalance between rates of ATPase-dependent Ca^{2+} uptake by the sarcoplasmic reticulum and rates of Ca^{2+} efflux and/or by pH-dependent activation of Ca^{2+} efflux from the sarcoplasmic reticulum (Hochachka, 1986; White and Somero, 1982). Once initiated, the pathological series of effects leading to necrotic cell death during prolonged hypothermia may be largely uncontrollable and analogous to the irreversible membrane injury and dissipation of ion gradients during anoxia (Fig. 1).

Little is known about the prolonged effects of cold exposure on cellular ion homeostasis in cold-tolerant animals. However, recent studies on frogs hibernating for up to 4 months reveal that these animals suppress their aerobic metabolic rate to 50 % of that seen in the resting air-breathing animal at 3 °C (Boutilier et al., 1997; Donohoe et al., 1998). During this time, a 30 % decrease in skeletal muscle Na^+/K^+ pump activity is accompanied by reduced Na^+ influx and K^+ efflux across the sarcolemma, the latter being mediated by ATP-sensitive K^+ (K_{ATP}) channels (Donohoe et al., 2000). The lowered rates of passive ion flux are coincident with reduced transmembrane ion gradients for [Na^+] and [K^+], which may also lower the energy costs of the skeletal muscle Na^+/K^+-ATPase. The ability of the skeletal muscle to maintain its resting membrane potential, coincident with decreased Na^+/K^+ pump activity and lowered membrane permeability, provides evidence of functional channel arrest as an energy-sparing strategy during hibernation in the cold-submerged frog (Donohoe et al., 2000).

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